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On May 29, 2003

TOWNSEND and ~~TOWNSEND~~ and CREW LLP

By: (Paula Faulk Hurley) Paula Faulk Hurley

PATENT
Attorney Docket No.: 2307AA-031220US
Client Ref. No.: 90-160-5

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:

Steinunn Baekkeskov *et al.*

Application No.: 08/838,486

Filed: April 7, 1997

For: IMPROVED METHODS FOR THE DIAGNOSIS AND TREATMENT OF DIABETES

Examiner: G. Ewoldt

Art Unit: 1644

DECLARATION OF STEINUNN BAEKKESKOV

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

I, Steinunn Baekkeskov, state as follows:

(1) I am Professor of Medicine and Microbiology/Immunology, and Horan Markarian Chair of Diabetes at the University of California, an assignee of the above-captioned application. A copy of my curriculum vitae is attached as Exhibit A. I have actively conducted research in diabetes for over twenty years. I regularly read the scientific literature, particularly that relating to diabetes, attend scientific meetings, and am conversant with the view of many colleagues.

(2) I have reviewed the above-captioned application of which I am a co-inventor and have followed the prosecution history thereof. I understand that the priority date of the application is September 7, 1990.

(3) The application is in large part premised on the discovery that glutamic acid decarboxylase (GAD) is a component of a pancreatic beta cell 64 kDa antigen that is a major autoantigen in insulin dependent diabetes mellitus (IDDM) (also known as type 1 diabetes). The application discloses administering GAD to a patient to inhibit or prevent IDDM. Administration of GAD induces tolerance to the 64 kDa autoantigen, thereby inhibiting or preventing further destruction of beta pancreatic cells, and the clinical symptoms of IDDM that eventually result from this destruction.

(4) When an antigen is administered to a subject, it can induce either a tolerogenic or an immune response depending on the regime with which it is administered. The application teaches that care should be taken not to potentiate an immune response that would exasperate ß cell destruction. Based on my knowledge of the scientific literature, general principles for achieving a tolerogenic response rather than a harmful immunogenic response were within the state of the art as of September 1990. For example, standard immunology textbooks available at the priority date of the invention discuss how either low or high dosages of antigen favor a tolerogenic response, whereas intermediate dosages favor an immunogenic response (Benjamini & Leskowitz, Immunology: A Short Course (Liss, 1988) at p. 255-256; Golub, The Cellular Basis of the Immune Response (2nd ed. Sinauer, 1981) at page 291). These textbooks also discuss how the use of unaggregated antigen favors a tolerogenic response. Induction of antigen specific tolerance had been used successfully in numerous studies to suppress or prevent autoimmune disease in animal models ((Cremer et al., Collagen induced arthritis in rats: antigen-specific suppression of arthritis and immunity by intravenously injected native type II collagen. J. Immunol. 131, 2995-3000 (1983); Scherer et al., Control of cellular and humoral immune responses by peptides containing

T cell epitopes. Cold Spring Harbor Symp. Quant. Biol. 54, 497-504, 1989; Nagler-Anderson et al., Suppression of type II collagen induced arthritis by intragastric administration of soluble type II collagen. Proc. Natl. Acad. Sci. USA 83, 7443-7446 (1986); Higgins and Weiner. Suppression of experimental autoimmune encephalomyelitis by oral administration of myelin basic proteins and its fragments. J. Immunol. 140, 440-445 (1988)). Given this guidance as to how to generate a tolerogenic response to ameliorate autoimmune disease, I believe scientists in the IDDM field would be able to use the knowledge of an identity of a target autoantigen to devise conditions to obtain a tolerogenic response to prevent or delay disease as of September 1990.

(5) This expectation has been confirmed by numerous reports in the scientific literature in which administration of GAD has been shown to induce tolerance in NOD mice and prevent IDDM (see *e.g.*, Tisch *et al.*, *Nature* 366, 71-75 (1993); Kaufman, *Nature* 366, 69-71 (1993), Tian *et al.*, *Nature Medicine* 12, 1348 (1996), Peterson *et al.*, *Diabetes* 44, 1478 (1994), and Pleau *et al.*, *J. Immunol. Immunopath.* 76, 90-95 (1995)). Several different parenteral routes of administration have successfully been used (see Harrison, *Molecular Medicine* 1, 722-727 (1994)).

(6) The NOD mouse is a good model of the major type of IDDM in which human patients develop autoantibodes and T cells to GAD, because NOD mice also develop autoantibodies and T cells to GAD (see Tisch *et al.*, *Nature* 366, 72-75 (1993) at *e.g.*, p. 21, column 1, first paragraph). The NOD mouse is a genetic strain of mouse that spontaneously develops autoimmunity to GAD, and subsequently symptoms of IDDM, in a manner similar to development of IDDM in humans. Positive results in the NOD mouse have been used as evidence to support human clinical trials of a number of drugs to treat IDDM. For example, human clinical trial of humanized OKT3 to treat IDDM is underway following a showing that such an antibody reversed hyperglycemia in NOD mice (see attached summary of the trial and Herold et al., New Engl. J. Med. 346, 1692-1698 (2002)). Similarly, a human clinical trial of alpha interferon is underway following

a showing that ingestion of alpha interferon prevents diabetes in a NOD mouse (see attached summary of trial). Most importantly, the results using GAD to induce tolerance and prevent diabetes in the NOD mouse have been used as evidence to support human clinical trials of a GAD vaccine to treat human type II diabetic patients (non-insulin dependent). These patients are treated with oral medication, but have autoantibodies to GAD, demonstrating that they are experiencing autoimmune destruction of ß cells and are therefore likely to become insulin dependent. The vaccine has been shown to be safe. The results of phase II of the clinical trials, which may provide an indication of the efficacy of the vaccine in preventing patients from becoming insulin dependent, will be announced at the American Diabetes Association annual meeting in New Orleans, June 13-17, 2003 (see attached summaries of trial).

(7) By contrast, the BB rat is not such a close model of IDDM in humans or other organisms that develop antibodies to GAD. The BB rat bears a genotype that results in spontaneous development of lymphocytopenia and clinical symptoms similar to those of IDDM. However, lymphocytopenia is not found in human IDDM. Furthermore, unlike the NOD mouse, and unlike most humans, the BB rat does not develop autoimmunity to GAD (see Petersen *et al.*, Autoimmunity 25, 129-138 (1997) at p. 134, col. 1). Because the BB rat does not develop autoantibodies to GAD, there is no reason to expect that therapeutic intervention with GAD would have any effect in the BB rat. Therefore, lack of such an effect in the BB rat, cannot be extrapolated to humans or other animals in which autoantibodies to GAD are present.

(8) In my opinion, the above evidence shows that a tolerogenic response has been obtained to GAD in mouse model of IDDM that is protective for IDDM and is predictive of similar response in humans. In my opinion, the evidence further shows this response was obtainable based on the teaching of the specification and common knowledge in the field as of September 1990.

(9) I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Respectfully submitted,

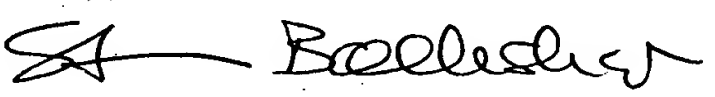

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Exhibit A

STEINUNN BAEKKESKOV

Curriculum Vitae

Affiliation Professor of Medicine and Microbiology/Immunology
Horan Markarian Chair of Diabetes

Address: Hormone Research Institute/Diabetes Center, Box 0534
University of California, San Francisco
San Francisco, CA 94143-0534

EDUCATION

1976: Candidatus Scientarum, (M. Sc./Ph.D.degree) in Biochemistry from the University of Copenhagen, Denmark.

1984: Licentiata scientarum, (Ph.D. degree) in Immunology from the University of Copenhagen, Denmark

PROFESSIONAL AND RESEARCH EXPERIENCE

1973-1975: Thesis student, Department of Chemistry, The Carlsberg Laboratory, Copenhagen. Isolation characterization and chemical modification of enzymes from *Saccharomyces cerevisae*. Thesis: Characterization and chemical modification of glucose-6-phosphate dehydrogenase from Brewers yeast.

1976 Lecturer in Biochemistry, Department of Biochemistry, University of Copenhagen Medical School

1977-1979: Postdoctoral Fellow, Department of Biochemistry, International Laboratory for Research on Animal Diseases (ILRAD), Nairobi, Kenya. Isolation and characterization of membrane proteins and lipids of African trypanosomes.

1980-1982: Postdoctoral Fellow, Hagedorn Research Laboratory, Gentofte, Denmark. Research area: Immunology/cell and molecular biology of the pancreatic β-cell. The role of autoimmunity in the pathogenesis of insulin-dependent diabetes.

1982-1986: Staff Scientist, Hagedorn Research Laboratory.

1986-1989: Senior Staff Scientist, Hagedorn Research Laboratory (permanent position).
1987-1989: Member of a panel of 6 Senior Staff Scientist that formed the Directory Board of the Hagedorn Research Laboratory.

1989-1992: Assistant Professor, Department of Medicine, Department of Microbiology/Immunology, University of California, San Francisco

1992-1998 Associate Professor Department of Medicine, Department of Microbiology/Immunology, University of California San Francisco.

1998-present Professor of Medicine and Microbiology/Immunology, University of California San Francisco

1990-1992: Member of UCSF Graduate Program in Endocrinology
1992-date: Member of UCSF Graduate Program in Molecular Medicine in PIBS
1992-date: Member of UCSF Biomedical Sciences Graduate Program
1993-date: Member of UCSF Graduate Program in Immunology in PIBS
1994-date: Member of UCSF Graduate Program in Cell Biology in PIBS

AWARDS AND HONORS

1970-1973	P. Wulff's Foundation Scholarship
1973-1975:	Carlsberg Foundation Research Student Fellowship Award
1982-1984:	Juvenile Diabetes Foundation Fellowship Award
1984-1987:	Juvenile Diabetes Foundation Career Development Award.
1991-1993	NIH-Shannon Award
1997-current	Horan Markarian Chair of Diabetes

PUBLICATIONS

Original Articles in Reviewed Journals:

1. Rovis, L. and **Baekkeskov, S.** Subcellular fractionation of *Trypanosoma brucei*. Isolation and characterization of plasma membranes. Parasitology **80**, 507-524 (1980).

2. **Baekkeskov, S.**, Kanatsuna, T., Klareskog, L., Nielsen, D.A., Peterson, P.A., Rubenstein, A.H., Steiner, D.F., and Lernmark, A. Expression of major histocompatibility antigens on pancreatic islet cells. Proc. Natl. Acad. Sci. USA **78**, 6456-6460 (1981).

3. Steffes, M.W., Nielsen, 0., Dyrberg, T., **Baekkeskov, S.**, Scott, J., and Lernmark, A. Islet transplantation in mice differing in the I and S subregions of the H-2 complex. Transplantation **31**, 476-479 (1981).

4. **Baekkeskov, S.**, Nielsen, J.H., Marner, B., Bilde, T., Ludvigsson, J., and Lernmark, A. Autoantibodies in newly diagnosed diabetic children immunoprecipitate specific human pancreatic islet cell proteins. Nature **298**, 167-169 (1982).

5. Dyrberg, T., **Baekkeskov, S.**, and Lernmark, A. Specific pancreatic β-cell surface antigens recognized by a xenogenic antiserum. J. Cell Biol. **94**, 472-477 (1982).

6. Dyrberg, T., Nakhooda, A.F., **Baekkeskov, S.**, Lernmark, A., Poussier, P., and Marliss, E.B. Islet cell surface antibodies and lymphocyte antibodies in the spontaneously diabetic "BB" Wistar rat. Diabetes **31**, 278-181 (1982).

7. Kanatsuna, T., **Baekkeskov, S.**, Lernmark, A., and Ludvigsson, J. Immunoglobulin from insulin-dependent diabetic children inhibits glucose-induced insulin disease. Diabetes **32**, 520- 524 (1983).

8. **Baekkeskov, S.**, and Lernmark, A. Glucose stimulates the biosynthesis of a human pancreatic islet cell protein, detected by an antiserum against the human erythrocyte glucose transporter. FEBS Letters **157**, 331-335 (1983).

9. **Baekkeskov, S.**, Dyrberg, T., and Lernmark, A. Autoantibodies against an Mr 64K islet cell protein precede the onset of insulin-dependent diabetes in the BB-rat. Science **224**, 1348-1350 (1984).

10. **Baekkeskov, S.** and Lernmark, A. A β-cell glycoprotein of Mr 40,000 is the major rat islet cell immunogen following xenogenic immunization. Diabetologia **27**, 70-73 (1984).

11. Gerling, I., **Baekkeskov, S.**, and Lernmark, A. Islet cell and 64K autoantibodies are associated with plasma IgG in newly diagnosed insulin-dependent diabetic children. J. Immunol. **137**, 3782-3785 (1986).

12. **Baekkeskov, S.**, Landin, M., Kristensen, J.K., Srikanta, S., Bruining, G.J., Mandrup-Poulsen, T., de Beaufort, C., Soeldner, J.S., Eisenbarth, G., Lindgren, F., Sundquist, G., and Lernmark, A. Antibodies to a Mr 64,000 human islet cell antigen precede the clinical onset of insulin-dependent diabetes. J. Clin. Invest. **79**, 926-934 (1987).

13. Efrat, S., **Baekkeskov, S.**, Lane, D., and Hanahan, D. Coordinate expression of SV40 large T and p53 proteins in β-cells of transgenic mice harboring hybrid insulin-large T antigen genes. Embo J. **6**, 2699-2704 (1987).

14. Warnock, G.L., Ellis, D., Rajotte, R.V., Dawidson, I., **Baekkeskov, S.**, and Egebjerg J. Studies on the isolation and viability of human islets of Langerhans. Transplantation **45**, 957-963 (1988).

15. Christie. M., Landin-Olsson; M., Sundkvist, G., Dahlquist, G., Lernmark, A. & **Baekkeskov, S.** Antibodies to a Mr 64,000 islet cell protein in Swedish children with newly diagnosed type 1 (insulin dependent diabetes. Diabetologia **31**, 597-602 (1988).

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18. **Baekkeskov, S.**, Warnock, G., Christie, M., Rajotte, R.V., Mose-Larsen, P., and Fey, S. Revelation of specificity of 64k autoantibodies in IDDM serums by high-resolution 2D-gel electrophoresis. Unambiguous identification of 64k target antigen. Diabetes **38**, 1133-1141 (1989).

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20. Christie, M., Pipeleers, D., Lernmark, A., **Baekkeskov, S.** Cellular and subcellular localization of a Mr 64000 protein autoantigen in insulin-dependent diabetes. J. Biol. Chem. **265**: 376-381 (1990).

21. **Baekkeskov, S.**, Aanstoot, H.J., Christgau, S., Reetz, A., Solimena, M., Cascalho, M., Folli, F., Richter-Olesen, H. and De Camilli, P. Identification of the 64k autoantigen in insulin-dependent diabetes as the GABA-synthesizing enzyme glutamic acid decarboxylase. Nature **347**, 151-156 (1990).

22. Christgau, S., Schierbeck, H., Aanstoot, H.J., Aagaard, L., Begley, J.S., Kofod, H., Hejnaes, K. and **Baekkeskov, S.** Pancreatic β-cells express two autoantigenic forms of glutamic acid decarboxylase, a 65kDa hydrophilic form and a 64kDa amphiphilic form which can be both membrane-bound and soluble. J. Biol. Chem. **266**, 21257-21264 (1991).

23. Christgau, S., Aanstoot, H.J., Schierbeck, H., Begley, K., Tullin, S., Hejnaes, K. and **Baekkeskov, S.** Membrane anchoring of the autoantigen GAD_{65} to microvesicles in pancreatic β-cells by palmitoylation in the N-terminal domain. J. Cell Biol. **118**, 309-320 (1992).

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25. Richter, W., Shi, Y. and **Baekkeskov, S.** Autoreactive epitopes in glutamic acid decarboxylase defined by diabetes-associated human monoclonal antibodies are localized in the middle and C-terminal domains of the smaller form of glutamate decarboxylase. Proc. Natl. Acad. Sci., USA, **90**, 2832-2836 (1993).

26. Radvanyi, F., Christgau, S., **Baekkeskov, S.**, Jolicoeur, C. and Hanahan, D. Pancreatic β cells cultured from individual preneoplastic foci in a multistage tumorigenesis pathway: a potentially general

technique for isolating physiologically representative cell lines. Mol. Cell. Biol., 13, 4223-4232. (1993).

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36. Kash, S. F., Johnson, R.S., Tecott, L.H., Noebels, J.L., Mayfield, R.D., Hanahan, D., and **Baekkeskov, S.** Epilepsy in mice deficient in the 65 kDa isoform of glutamic acid decarboxylase. Proc, Natl. Acad. Sci. USA 94, 14060-14065 (1997).

37. Hensch, T. K., Fagiolini, M., Mataga, N., Stryker, M. P., **Baekkeskov, S.**, and Kash, S. F. Local GABA circuit control of experience-dependent plasticity in developing visual cortex. Science 282, 1504-1508 (1998).

38. Bridget, M., Cetkovic-Cvrlje, O'Rourke, R., Shi, Y., M., Narayanswami, S., Lambert, J., Ramiya, V., **Baekkeskov, S.**, and Leiter, E. Differential protection in two transgenic lines of NOD/Lt mice hyperexpressing the autoantigen GAD65 in pancreatic beta cells. Diabetes 47, 1848-1856 (1998).

39. Kash, S. F., Tecott, L. H., Hodge, C., and **Baekkeskov, S.** Increased anxiety and altered responses to anxiolytics in mice deficient in the 65 kDa isoform of glutamic acid decarboxylase. Proc. Natl. Acad. Sci. **96**, 1698-1703 (1999).

29. Schwartz, H., Chandonia, J-M., Kash, S. F., Tunnell, E., Domingo, A., Cohen, F., Banga, J. P., Madec, A.-M., Richter W., and **Baekkeskov, S.** High resolution epitope mapping and structural modeling of human glutamic acid decarboxylase 65. J. Mol. Biol. **287**, 983-999 (1999).

30. Kash, S., Condei, B., and **Baekkeskov, S.** GABA in brain and pancreas: Lessons from knock-out mice. Hormone and Metabolic Research **31**, 340-344, 1999.

31. Tian, N., Petersen C., Kash, S. F., **Baekkeskov, S.**, Copenhagen, D. and Nicoll, R. The role of the synthetic enzyme GAD65 in the control of neuronal GABA release. Proc. Natl. Acad. Sci. USA, **96**, 12911-12916, (1999).

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33. Roll, U., Turck, C.W., Gitelman, S.E., Rosenthal, S.M., Nolte, M.S., Masharani, U., Ziegler, A.-G., and **Baekkeskov, S.** Peptide mapping and characterization of glycosylation patterns of the glima 38 antigen recognized by autoantibodies in Type 1 diabetic patients. Diabetologia, **43**, 598-608 (2000).

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35. Kang, S-M., Braat, D., Schneider, D. B., O'Rourke, R. W., Lin, Z., Ascher, N. L., Dichek, D. J., **Baekkeskov, S.**, and Stock, P. G. A non-cleavable mutant of Fas ligand does not prevent neutrophilic destruction of islet transplants. Transplantation, **69**, 1813-1817, (2000).

36. Shi, Y., Kanaani, J., Menard-Rose, V., Ma, Y-H., Chang, P-Y, Hanahan, D., Grodsky, G., and **Baekkeskov, S.** Increased expression of glutamic acid decarboxylase and GABA in pancreatic β-cells impairs glucose-stimulated insulin secretion at a step proximal to membrane depolarization. Am. J. Physiol. Endocrinol. Metab. **279**, 684-694, (2000).

37. Wolfe, T., Bot, A., Hughes, A., Möhrle, U., Rodrigo, E., Jaume, J. C., **Baekkeskov, S.**, and von Herrath, M. Endogenous expression of autoantigens influence success or failure of DNA immunizations to prevent type 1 diabetes: addition of IL-4 increases safety. Eur. J. Immunol. **32**, 113-121. 2002.

38. Jaume, J. C., Parry, S. L., Madec, A.-M., Sønderstrup, G., and **Baekkeskov, S.** Suppressive effect of GAD65-specific autoimmune B lymphocytes on processing of T cell determinants located within the antibody epitope. J. Immunology **169**, 665-672, 2002.

39. Kanaani, J., El-Husseini, A. E-D., Aguilera-Moreno, A., Diacovo, J. M., Bredt, D. S., and **Baekkeskov, S.** A combination of three distinct trafficking signals mediates axonal targeting and presynaptic clustering of GAD65. J. Cell Biol. **158**, 1229-1238. 2002.

40. Hayakawa, N., Premawaedhana, L.D.K.E., Powell, M., Masuda, M.. Arnold, C., Sanders, J., Evans, M., Chen, S., Jaume, J. C., **Baekkeskov, S.**, Rees Smith, B. and Furmaniak, J. Isolation and characterization of human monoclonal autoantibodies to glutamic acid decarboxylase. Autoimmunity 35, 343-355, 2002.

Symposia Papers, Reviews, and Book Chapters:

1. Lernmark, A. and **Baekkeskov, S.** Islet cell antibodies - theoretical and practical implications. Diabetologia **21**, 431-435 (1981).

2. Lernmark, A., Bonnevie-Nielsen, V., **Baekkeskov**, S., Dyrberg, T., Kanatsuna, T., and Scott, J. Islet cell antibodies. **In**: Etiology and pathogenesis of insulin-dependent diabetes mellitus, eds.: J.M. Martin, R.M. Ehrlich, and F.J. Holland, Raven Press, N.Y., pp. 61-71 (1981).

3. Baekkeskov, S., Dyrberg, T., Kanatsuna, T., Lernmark, A., Takei, I., and Soderstrum, K. The significance of ICSA in IDDM among Caucasians. **In**: Proceedings of the International Symposium on Clinico-Genetic Genesis of Diabetes Mellitus, Kobe, Feb. 11-12, 1982, eds.: G. Mimura, S. Baga, Y. Goto, and J. Kobberling, Excerpta Medica, Amsterdam, pp. 137-141 (1982).

4. **Baekkeskov**, S. and Lernmark, A. Rodent islet cell antigens recognized by antibodies in sera from diabetic patients. Acta Biol. Med. Germ. **41**, 1111-1115 (1982).

5. Brogren, C.H., **Baekkeskov**, S., Dyrberg, T., Lernmark, A., Marner, B., Nerup, J., and Papadopoulos, G.K. Role of islet cell antibodies in the pathogenesis of type I diabetes. **In**: Diabetes and Immunology: Pathogenesis and Immunotherapy, eds.: H. Kolb, G. Schernthaner, and F.A. Gries, Hans Hubert Publishers. Berlin, pp. 65-78 (1983).

6. **Baekkeskov**, S. Radiolabelling and immunoprecipitation of islet cell antigens. **In**: Methods in Diabetes Research, Volume I, part A, eds.: J. Larner and S.L. Pohl, John Wiley & Sons, Ltd., New York, pp. 129-140 (1984).

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DIAMYD MEDICAL

Press Release
Stockholm, April 11, 2003

Diamyd Medical to present Phase II results with diabetes vaccine in June

Diamyd Medical (O-list) is developing a GAD vaccine for insulin-dependent diabetes. The company plans to present results from its Phase II trial with the vaccine during the American Diabetes Association Congress in New Orleans, June 13-17, 2003. The study's main aims are, in the first place, to investigate the safety of the vaccine and in the second place to obtain an indication of the vaccine's efficacy. It is Diamyd Medical's ambition, if the Phase II trial is successful, to seek co-operation with an established pharmaceutical company for further comercialization of the vaccine and in doing so to achieve a positive cash flow.

Diamyd Medical has been conducting a Phase II study since May 2001 on 48 orally treated diabetes patients who have antibodies to GAD. The GAD antibodies indicate that an autoimmune process is underway, that eventually will destroy the patient's insulin-producing cells by which time patients will be dependent on daily insulin injections. The Diamyd vaccine is intended to prevent this development so that patients continue to produce their own insulin. This Phase II study, the results of which are expected to be available in June, is aimed in the first place to investigate if the vaccine is safe to administer as well as to provide an indication of the vaccine's effect at various dose levels. The study is double-blind and placebo controlled, that is nobody knows who has received the active vaccine or the placebo.
"We are working to be able to break the code in June and present the results of the study during the American diabetes conference, which this year is being held in New Orleans June 13-17," says Anders Essen-Möller. "This event is usually attended by some 8,000 doctors and scientists and we hope to reach out with our results to them."

About Diamyd Medical:
Diamyd Medical's business idea is to identify and develop pharmaceutical projects up to and including Phase II. At present Diamyd Medical is running a number of GAD-based development projects and has the licensed rights for this from universities in the US. The Company's project that has come farthest is a vaccine for insulin-dependent diabetes.
The projects will then be sold or licensed to major pharmaceutical companies for further commercialization. The development and marketing of related diagnostic tests and substances takes place in parallel to promote contact with researchers and prepare the market for the impending pharmaceuticals.

For further information, please contact:
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Phone: +46 8-661 00 26, +46 8-661 12 25,
fax: +46 8-661 63 68, or via e-mail: info@diamyd.com

DIAMYD MEDICAL

Press Release
Stockholm, April 28, 2003

Major potential for Diamyd's diabetes vaccine

Diamyd Medical (O-list) reports that the last patient samples have been taken today in the ongoing six month's Phase II study with the GAD-based diabetes vaccine, Diamyd™. Diamyd Medical is planning to present the results from the study at the American Diabetes Association scientific congress in the US between June 13 and 17. The first application of the vaccine is seen to be preventing diabetes patients being treated with insulin in tablet form from becoming dependent on injections. Further applications are expected to be the prevention of insulin dependency in children and young people who run the risk of developing the illness and increasing the survival capability of insulin producing cells after transplantation.

The first diabetes patient was injected with the Diamyd diabetes vaccine in May 2001. Since then, a total of 47 patients have been included in Diamyd Medical's clinical Phase II study that has been carried out in both Malmö and Stockholm. Each patient has visited the hospital ten times during a six-month period so that samples could be taken. Extensive analysis of the samples have been carried out by experts in the US, the UK and Sweden. Apart from the safety aspects of the vaccine, both metabolic and immunological parameters are thoroughly monitored aimed at obtaining an indication of the vaccines function and efficacy. "Today another milestone has been passed with the last four patient samples being taken in the six-month study," says CEO Anders Essen-Möller. "We look forward with great interest to the results that are expected to be published in June at the American Diabetes Association conference in New Orleans."

The first category for the vaccine is seen to be those diabetes patients with antibodies against GAD who are being treated with tablets. The annual market for this patient category is estimated to be SEK 5-10 billion. There are similar markets if the vaccine can be developed to prevent insulin-dependent diabetes in children and young people.

About Diamyd Medical:
Diamyd Medical's business idea is to identify and develop pharmaceutical projects up to and including Phase II. At present Diamyd Medical is running a number of GAD-based development projects and has the licensed rights for this from universities in the US.

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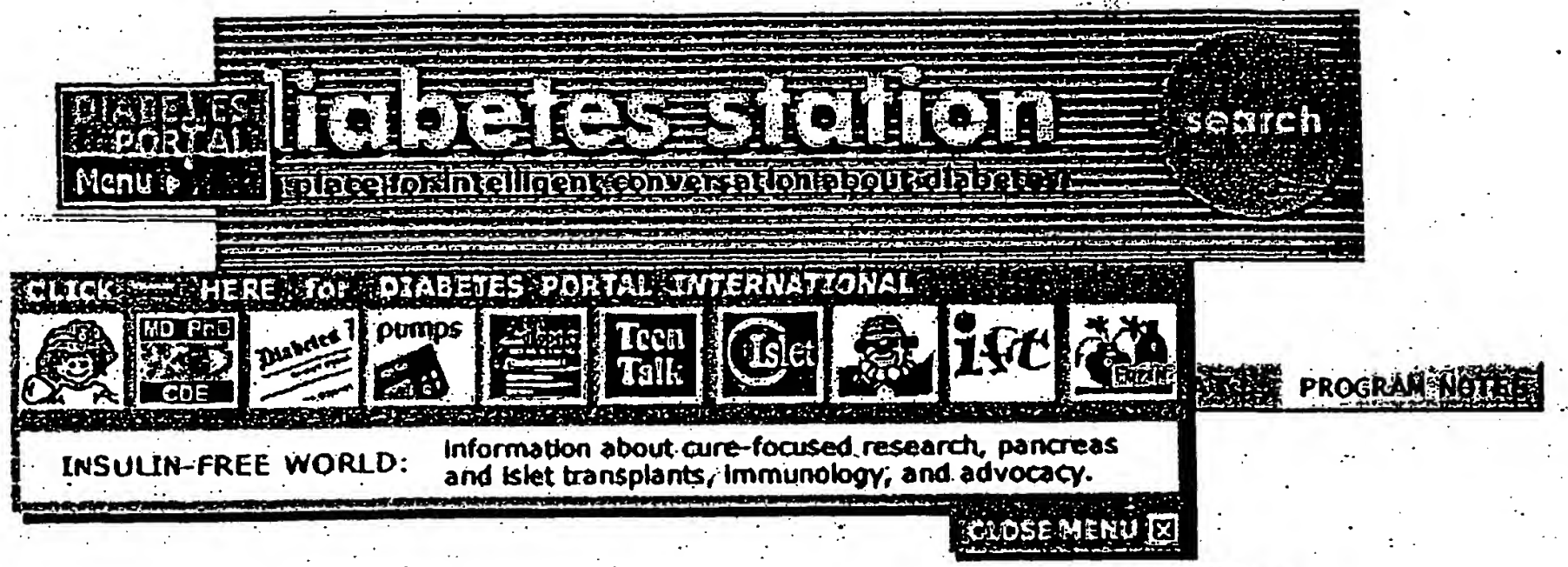
DIAMYD MEDICAL



DO YOU WANT TO TALK ABOUT IT?

NEW CLINICAL TRIAL IN NEWLY DIAGNOSED TYPE 1 DIABETES HOUSTON, TEXAS

THE UNIVERSITY OF TEXAS DIABETES RESEARCH GROUP NEWSLETTER presents new information on studies of oral (ingested) type I interferon. The Endocrinology Divisions in both Internal Medicine and Pediatrics are now recruiting newly diagnosed type 1 diabetes patients in a phase II randomized, double-blind, parallel-design clinical trial to determine whether ingested (oral) human recombinant IFN-a will prolong the 'honeymoon' period. We have demonstrated that ingested IFN-a prevents type 1 diabetes in the NOD mouse. Ingested IFN-a also prolongs the 'honeymoon' period in newly diagnosed type 1 diabetics in phase I open label clinical trial recently completed here at UT-Houston. The natural history of type 1 diabetes is unique for a phase frequently referred as the "honeymoon", a period in which the insulin need becomes minimal and glycemic control improves. The b cell partially recovers. However, as with all honeymoons, they end and the patient becomes completely insulin-deficient. The general consensus of the international diabetes community is to test potential preventive therapies for type 1 diabetes in newly diagnosed patients. Prolongation of the honeymoon as the reversal of the disease is considered a positive result.

Entry criteria include male or female type 1 diabetes patients requiring insulin within one month of diagnosis between the ages of 3-25 without concurrent diseases. Eighty eligible patients will be randomized into one of two treatment arms - the active treatment arm will ingest 30,000 units IFN-a daily and the non-active treatment arm will ingest placebo (saline) for one year.

Prior to enrollment into the study (within 1 month of diagnosis), patients will be evaluated in the UT University Clinical Research Center at Hermann Hospital with a complete medical exam and routine blood tests. Patients

will be seen monthly for the first three months, and every three months thereafter. Primary outcome measures will be a 30% increase in C-peptide levels released after Sustacal stimulation at 3, 6, 9, and 12 months after entry. If successful, this will lead to a larger and longer phase III trial of prevention of type 1 diabetes in high risk patients.

We appreciate your help in referring patients to our Diabetes Research Group. Your efforts allow patients the opportunity to be involved in cutting edge clinical trials. There is no charge to your patients. Patients will continue to be followed by their private endocrinologist for optimization of glycemic control during the course of the study. This trial will require trips to Houston at entry and at months 1, 2, 3, 6, 9, and 12 for testing.

If you have or know of patients that might wish to participate in this clinical trial outlined above, please call any of the numbers below.

Staley A. Brod, MD Principal Investigator - 713 500-7046 or 713 500-7050, Fax:713-500-7041 (PI)
Phil Orlander, MD Adult Endocrinology - Co- Principal Investigator 713-500-6646
Victor Lavis, M.D. Adult Endocrinology
Patrick Brosnan, M.D. Pediatric Endocrinology - 713-500-5646
Lucie Lambert, Asst. to Dr. Brod 713 500-7050.
The University of Texas - Houston.
Department of Pediatrics, Internal Medicine, and Neurology (Immunology)
6431 Fannin St
Houston, Texas 77030

LINKS ABOUT THIS INVESTIGATOR AND CLINICAL TRIAL -

About Dr. Brod
More about Dr. Brod - 1981
How to participate in a study at the University of Texas - Houston

DO YOU WANT TO TALK ABOUT IT?

The NEW ENGLAND JOURNAL of MEDICINE

ORIGINAL ARTICLE

◂ Previous Volume 346:1692-1698 May 30, 2002 Number 22 Next ▸

Anti-CD3 Monoclonal Antibody in New-Onset Type 1 Diabetes Mellitus

Kevan C. Herold, M.D., William Hagopian, M.D., Ph.D., Julie A. Auger, B.A., Ena Poumian-Ruiz, B.S., Lesley Taylor, B.A., David Donaldson, M.D., Stephen E. Gitelman, M.D., David M. Harlan, M.D., Danlin Xu, Ph.D., Robert A. Zivin, Ph.D., and Jeffrey A. Bluestone, Ph.D.

ABSTRACT

Background Type 1 diabetes mellitus is a chronic autoimmune disease caused by the pathogenic action of T lymphocytes on insulin-producing beta cells. Previous clinical studies have shown that continuous immune suppression temporarily slows the loss of insulin production. Preclinical studies suggested that a monoclonal antibody against CD3 could reverse hyperglycemia at presentation and induce tolerance to recurrent disease.

Methods We studied the effects of a nonactivating humanized monoclonal antibody against CD3 — hOKT3γ1(Ala-Ala) — on the loss of insulin production in patients with type 1 diabetes mellitus. Within 6 weeks after diagnosis, 24 patients were randomly assigned to receive either a single 14-day course of treatment with the monoclonal antibody or no antibody and were studied during the first year of disease.

Results Treatment with the monoclonal antibody maintained or improved insulin production after one year in 9 of the 12 patients in the treatment group, whereas only 2 of the 12 controls had a sustained response (P=0.01). The treatment effect on insulin responses lasted for at least 12 months after diagnosis. Glycosylated hemoglobin levels and insulin doses

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were also reduced in the monoclonal-antibody group. No severe side effects occurred, and the most common side effects were fever, rash, and anemia. Clinical responses were associated with a change in the ratio of CD4+ T cells to CD8+ T cells 30 and 90 days after treatment.

Conclusions Treatment with hOKT3γ1(Ala-Ala) mitigates the deterioration in insulin production and improves metabolic control during the first year of type 1 diabetes mellitus in the majority of patients. The mechanism of action of the anti-CD3 monoclonal antibody may involve direct effects on pathogenic T cells, the induction of populations of regulatory cells, or both.

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- Herold, K. C.
- Bluestone, J. A.
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COLLECTIONS

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Source Information

From the Naomi Berrie Diabetes Center and the Department of Medicine, Division of Endocrinology, College of Physicians and Surgeons, Columbia University, New York (K.C.H., E.P.-R., L.T.); Pacific Northwest Research Institute, Seattle (W.H.); the University of Chicago, Chicago (J.A.A.); the University of Utah, Salt Lake City (D.D.); the Departments of Pediatrics (S.E.G.) and Medicine (J.A.B.), University of California at San Francisco, San Francisco; the National Institute of Diabetes and Digestive and Kidney Diseases, Bethesda, Md. (D.M.H.); and the R.W. Johnson Pharmaceutical Research Institute, Raritan, N.J. (D.X., R.A.Z.).

Address reprint requests to Dr. Herold at Columbia University, 1150 St. Nicholas Ave., New York, NY 10032, or at kh318@columbia.edu.

Full Text of this Article

Related Letters:

Anti-CD3 Monoclonal Antibody in New-Onset Type 1 Diabetes Mellitus
Killestein J., Herold K. C., Bluestone J. A.
Full Text
N Engl J Med 2002; 347:1116-1117, Oct 3, 2002. **Correspondence**

This article has been cited by other articles:

- Herold, K. C., Burton, J. B., Francois, F., Poumian-Ruiz, E., Glandt, M., Bluestone, J. A. (2003). Activation of human T cells by FcR nonbinding anti-CD3 mAb, hOKT3{gamma}1(Ala-Ala). *J. Clin. Invest.* 111: 409-418 [Abstract] [Full Text]
- Tang, Q., Smith, J. A., Szot, G. L., Zhou, P., Alegre, M.-L., Henriksen, K. J., Thompson, C. B., Bluestone, J. A. (2003). CD28/B7 Regulation of Anti-CD3-Mediated Immunosuppression In Vivo. *J Immunol* 170: 1510-1516 [Abstract] [Full Text]
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Immune Tolerance Netw

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Clinical Trial Research Summary

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A Phase II, multiple dose treatment of Type 1 diabetes with hOKT3γ-1(Ala-Ala

Principal Investigator: Kevan Herold, Columbia University

Abstract | Investigators | News | Background | Resources

Abstract

Objective: The objective of this proposal is to study the immunologic effects of humani non-binding anti-CD3 mAb on immune responses associated with Type 1 diabetes (T1DI develop this therapy to prevent the immune destruction leading to beta cell loss.

Basis/Rationale: Studies of the natural history of T1DM indicate that 100% of individu the disease still make detectable insulin even after the first year of diabetes but lose tht completely over the next 5 years. Retention of the ability to produce any insulin endoge results in improved clinical control of the disease, and therefore, reduced secondary complications. In the NOD mouse, anti-CD3 mAb reversed hyperglycemia after presenta hyperglycemia, induced long lasting protection from disease in the absence of continuo treatment, and prevented recurrent diabetes in recipients of islet allografts. Pre-clinical FcR non-binding anti-CD3 mAb suggest that the mAb selectively anergizes activated Th possibly by delivering an altered TCR signal. Th1 cells are thought to be involved in T1D are most prevalent in the islet at the late stages of the disease, thus suggesting the bas efficacy of anti-CD3 mAb even after presentation. We have been conducting a Phase I/I this agent in patients with new onset T1DM. The drug has been well tolerated and does the toxicities of OKT3. Our studies have suggested a dosing regimen appropriate for Phi trials and mechansms that may account for the mAb effect.

Significance: The trial formally tests the hypothesis, that in man, T1DM is mediated by lymphocytes. This drug may be of valuefor treatment of T1DM and prevention of its rec islet allografts. Relevance of Immune Tolerance: In mouse studies, the drug induces tol T1DM. This study will test the same in man, and develop a protocol that will maintain to islet cells.

Clinical Protocol Summary: In this Phase II protocol, the mAb will be administered or 1, 2, or 3 occasions during the first 1 1/2 years of disease. This protocol differs from the Phase I, single treatment protocol in that repeated administration of the mAb is utilized the effects of the mAb in a manner analogous to repeated administration of a vaccine. C responses of the treated groups (n=24 in each) will be compared to untreated patients.

Mechanistic Studies: The planned mechanistic studies, included in this proposal are sp questions pertaining to the immunologic effects in diabetes, and will determine how the works and how to best utilize it for treatment. The studies test three mechanisms to acc the actions of the drug. First, that pathogenic T cells are deleted from the repertoire by treatment. Second, that the drug anergizes specific populations of T cells, most likely Th cells including those cells responsible for islet antigen recognition. Third, that subpopula cells activated by mAb (i.e. CD69+ or CD25+) represent a regultory population that ma the function and/or effects of autoimmune effector cells. In addition to these studies, sa and clinical information from this trial should facilitate studies using tolerance assays of

Participating Investigators

- *Kevan Herold,* Naomie Berrie Diabetes Center, Columbia University

- *Jeffrey A. Bluestone*, THe Diabetes Center at UCSF
- *Michael H. Dosch*, University of Toronto, Canada
- *Peter Gottlieb*, Barbara Davis Diabetes Center
- *Carla Greenbaum*, Virginia Mason Center
- *William Hagopian*, Pacific Northwest Research Institute
- *David Harlan*, National Institutes of Health
- *Andrew Muir*, University of Florida, Gainesville
- *Gerry Nepom*, Virginia Mason Center
- *Jerry Palmer*, University of Washington

News & Recent Developments

- Anti-CD3 Monoclonal Antibody in New-Onset Type 1 Diabetes Mellitus - *NEJM* [go→]
- Anti-CD3 Monoclonal Antibody Slows New-Onset Type 1 Diabetes - *Medscape* [go→]

Background Articles

- Prevention of autoimmune diabetes with nonactivating anti-CD3 monoclonal antibody *Diabetes* [go→]
- CD3 antibody-induced dominant self tolerance in overtly diabetic NOD mice - *J Immu* →]
- Anti-CD3 Antibody Induces Long-Term Remission of Overt Autoimmunity in Nonobes Diabetic Mice - *PNAS* [go→]
- OKT3γ(ala-ala) delivers partial TCR signal- *-J Immunol* [go→]

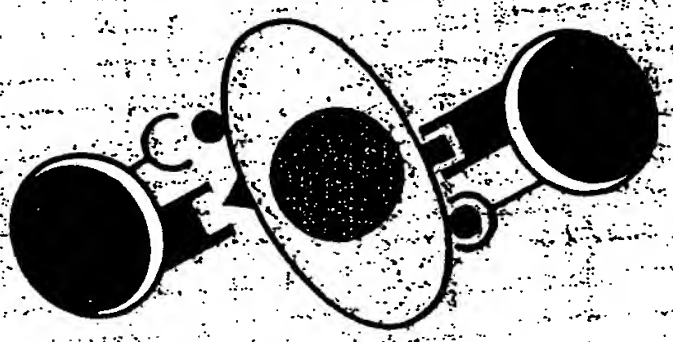
Resources & Interesting Links

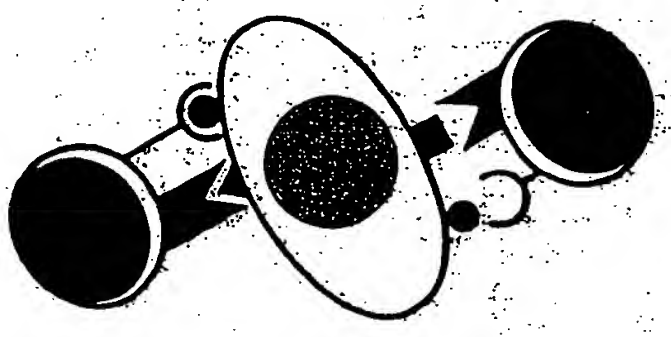
- Tolerogenic strategies to halt or prevent type 1 diabetes Nat Immunol [go→]

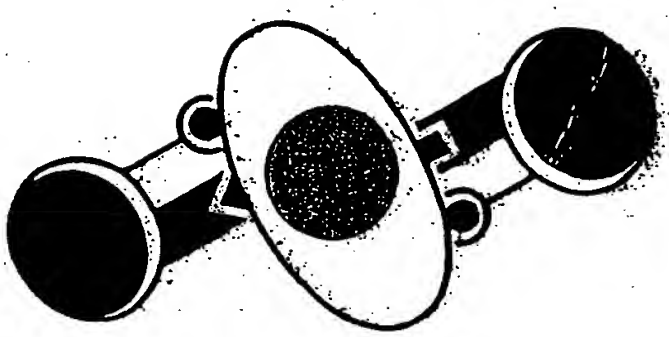
Edward S. Golub

THE CELLULAR BASIS OF THE IMMUNE RESPONSE

SECOND EDITION







THE COVER

While it is basically true that "one cannot tell a book by its cover," the cover sometimes tells a great deal about the book. The cover logo for the first edition of *The Cellular Basis of the Immune Response* summarized the main theme of the book and the status of cellular immunology in 1977. Helper and effector cells, each reactive to different portions of the antigen molecule, were shown interacting to bring about an immune response.

By 1981 the situation has become more complex and the cover logo of the present edition reflects these changes. The emphasis now is on the T-cell reacting with the macrophage through two sets of receptors. One set of receptors is for antigen and the other is directed against self-MHC antigens. This reflects the central role of the MHC in immune reactions and the need for autoreactivity against MHC.

For Jonathan and Mark . . .
who make it all worthwhile

Library of Congress Cataloging in Publication Data

Golub, Edward S 1934–
The cellular basis of the immune response.

Includes bibliographical references and index.
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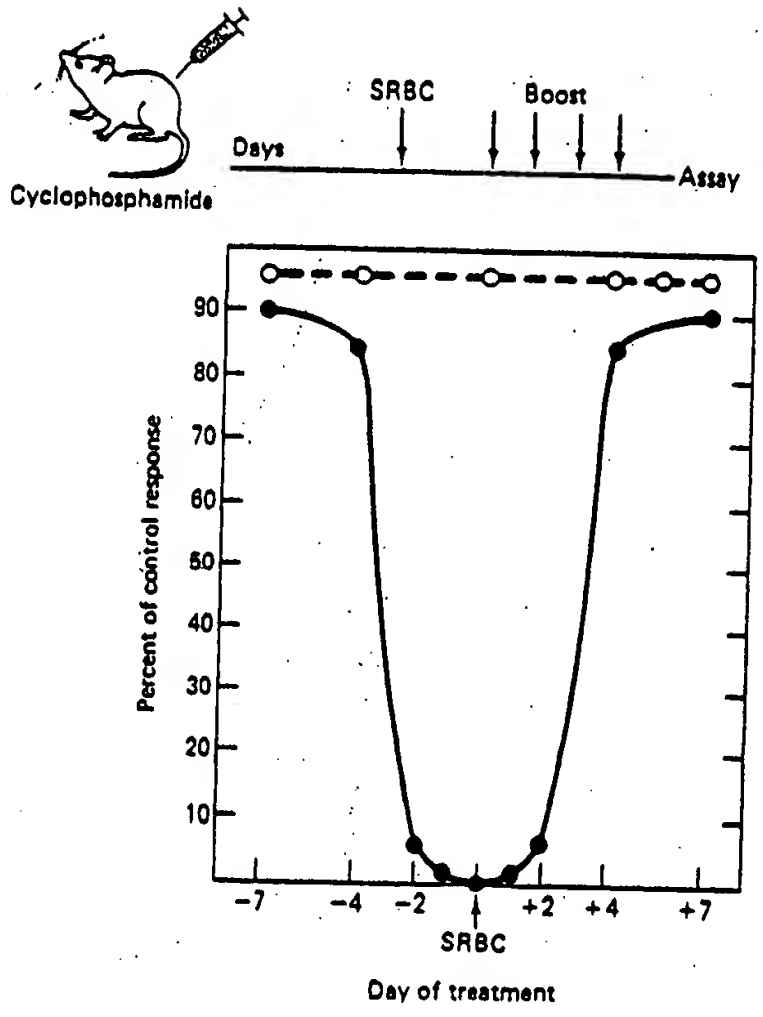

were treated with cyclophosphamide either before or after iving SRBC as tolerogen. SRBC were injected several times, and SRBC assayed on day 35. Anti-SRBC response was diminished, esponse to non-cross-reacting antigen was normal. [From Aisen- (1967). *J. Exp. Med.* 125, 833.]

e, treatment with the drug 7 or 14 days before SRBC injection 10 effect on subsequent SRBC responses. However, treatment for row time span of 3 days before or 3 days after SRBC treatment ted in loss of ability to produce anti-SRBC while the ability to nd to an unrelated antigen was unimpaired. This shows that a of specific tolerance was established to SRBC when the mimetic drug was used at the time that antigen was introduced. ar results have been obtained with BSA in the rabbit using not adiomimetic drugs but nonlethal doses of X-irradiation.

Antigen Form

The form in which the antigen is injected can also be important in inducing immunity or tolerance. If the antigen is in a form to which the animal cannot respond by producing antibody or a cell-mediated response, a state of tolerance is often established. We have already seen that the aggregate-free form of the antigen induces tolerance. Another example of this is seen when mice are injected with the hapten DNP on a carrier which is nonimmunogenic such as poly-D-GL. This is a random polymer of D-glutamic acid and D-lysine. The L-polymer of poly-GL is immunogenic, but the D-polymer is not. When mice pretreated with the DNP-D-GL polymer are challenged with DNP on the immunogenic carrier KLH, they fail to produce antibody to DNP but make normal amounts of antibody to an unrelated antigen. Thus the DNP-D-GL acts as a tolerogen.

Antigen Concentration

Another means of inducing tolerance is to treat animals with *extremes of antigen concentration*. In a phenomenon known as *immune paralysis* discovered by Felton in 1934, mice are treated with either 100 or 10μg pneumococcal polysaccharide (abbreviated S III). The mice which receive the 10μg S III are protected when challenged with virulent pneumococcus organisms, but all those pretreated with 100μg die of the disease. In this case a low dose immunized the mice, but a high dose did not. It was thought that the high dose of antigen "paralysed" the immune system. We now think of this as a form of tolerance in which there is failure of lymphocytes to respond to antigen.

A rather surprising observation was made when *extremely low doses* of protein antigen were used to induce tolerance. In the heroic experiment of Mitchison shown in Figure 4 groups of mice were injected with varying doses of soluble BSA three times a week for up to 16 weeks. In this way the effect of dose and time could be determined. The animals were then challenged with the immunogenic form of BSA, and anti-BSA antibody titers were determined. When the data was plotted, it was found that high doses of tolerogen induced tolerance (as expected) but that very low doses also induced tolerance. This phenomenon is called LOW ZONE TOLERANCE and has now been observed with several antigens. Treatment with middle doses of BSA primed the mice.

Antibody-Induced Tolerance

Tolerance can also be induced by administrating *antibody*.

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COLLAGEN-INDUCED ARTHRITIS IN RATS: ANTIGEN-SPECIFIC SUPPRESSION OF ARTHRITIS AND IMMUNITY BY INTRAVENOUSLY INJECTED NATIVE TYPE II COLLAGEN[1]

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Collagen-induced arthritis (CIA) developed in 70 to 90% of rats immunized with heterologous type II collagen. CIA was reduced to 0 to 18% when rats were injected i.v., i.e., pretreated, with 1 mg of soluble native type II collagen before immunization. Concomitant with the suppression of CIA were significant suppression of IgM, IgG, and delayed-type hypersensitivity (DTH) responses to type II collagen. Suppression of CIA and immunity to collagen was antigen-specific, related to dose and route of administration, and occurred only when 1 mg of collagen was injected i.v. either 32, 7, or 4 days before, or 7 days after immunization. Once CIA was established, however, neither arthritis nor immunity could be suppressed.

To determine if adjuvant-induced arthritis (AIA), like CIA, could be suppressed by i.v. pretreatment with type II collagen, rats were given 1 mg of type II collagen or PBS i.v. before injection with mycobacteria and oil. AIA was not suppressed, and arthritis appeared in both groups at a similar incidence and severity. Sera from 26 rats with severe AIA that was collected between days 14 and 35 after injection were assayed for IgG to homologous rat type II collagen and were found to be negative.

These findings further support the hypothesis that CIA in rats is mediated by immunity to type II collagen and also suggest that CIA and AIA have different primary pathogenic mechanisms.

Collagen-induced arthritis (CIA)[3] is an experimental model of inflammatory polyarthritis that can be induced in 70 to 90% of susceptible rats (1–18) or mice (19–21) by sensitizing them with heterologous native type II collagen. In addition to arthritis, approximately 14% of rats also develop inflammatory auricular chondritis that histologically resemble the lesions found in relapsing polychondritis (8–10).

Although the exact pathogenic mechanism(s) remain to be defined, substantial evidence exists linking CIA with the immune response to type II collagen. Investigators from this laboratory (1–4) and others (5–7) have shown that sera from rats with CIA contain significantly higher titers of antibody to type II collagen than sera from sensitized rats that failed to develop disease. Furthermore, we have shown a temporal relation between the onset of CIA and the presence of circulating IgM and IgG immunoglobulins and lymphocytes reactive with native type II collagen (4). Additional evidence supporting an immune-mediated pathogenesis of CIA includes the adoptive transfer of arthritis to naive recipients (11), abrogation of disease by depleting serum complement (C) with cobra venom (12), modulation of CIA with immunostimulatory and immunosuppressive agents (13), suppression of CIA by i.v. injection of collagen-coated spleen cells (14), and most recently the passive transfer of CIA to naive recipients by using specific IgG prepared from sera of rats with acute arthritis (15, 16).

Reported here are studies showing that a single injection of soluble type II collagen, given i.v., before or shortly after intradermal (i.d.) challenge with an arthritogenic preparation of type II collagen, was highly effective and specific in suppressing CIA and immunity to type II collagen. In contrast, an i.v. injection of type II collagen had no effect on the incidence or severity of AIA. These findings further support the hypothesis that CIA in rats is mediated by immunity to native type II collagen and also suggest that CIA and AIA have different primary pathogenic mechanisms.

MATERIALS AND METHODS

Animals. Outbred female Wistar rats (Charles River Breeding Laboratories, Inc., Wilmington, MA or Harlan Sprague-Dawley, Indianapolis, IN) weighing between 100 and 125 gm were used for these studies. Rats from both suppliers developed CIA at the same incidence and produced virtually identical IgG responses to type II collagen. Rats were housed in groups of five in wire-bottomed metal cages and fed standard laboratory chow and water *ad libitum*.

Collagen preparation. Native bovine (BII), chick (CII), and rat (RII) type II collagens were solubilized by limited pepsin digestion of fetal bovine cartilage, chick sterna, and rat chondrosarcoma tumor, respectively; and were purified as described (3). Native bovine type I collagen (BI) was solubilized by pepsin digestion of fetal bovine skin (1). Purity of collagen was determined by amino acid analysis, sodium dodecyl sulfate polyacrylamide gel electrophoresis, and uronic acid analysis.

Protocol to modify CIA by i.v. injection of solubilized type II collagen. To determine whether the incidence, severity, or duration of CIA could be modified by injection of soluble type II collagen without incomplete Freund's adjuvant (IFA), rats were injected i.v. with a solution of type II collagen in phosphate-buffered saline (PBS) pH 7.2. This procedure will herein be referred to as pretreatment. To determine the effect of pretreatment on the course of CIA, rats were immunized with BII. Immunization will be referred to as challenge. The dose of collagen given i.v. and time of pretreatment relative to challenge were varied depending on the design of the experiment.

Preparation of soluble antigens for i.v. injection. Solutions of collagen for i.v. injection were prepared as follows. Native CII, BII, or BI collagens were dissolved overnight at 4°C in 0.1 M acetic acid (1.2 mg/ml), dialyzed in PBS, and centrifuged at 100,000 × G for 30 min to remove any insoluble collagen.

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[2] Dr. Cremer is a Postdoctoral Fellow of the Arthritis Foundation. Requests for reprints should be addressed to him at the Veterans Administration Medical Center, Research Service (151), 1030 Jefferson Avenue, Memphis, TN 38104.
[3] Abbreviations used in this paper: AIA, adjuvant-induced arthritis; BI, native bovine type I collagen; BII, native bovine type II collagen; CII, native chick type II collagen; CIA, collagen-induced arthritis; CMI, cell-mediated immunity; DTH, delayed-type hypersensitivity; ELISA, enzyme-linked immunosorbent assay; i.d., intradermal; MAI, maximum arthritis index; OVA, ovalbumin; RII, native rat type II collagen; s.c., subcutaneous.

The concentration of collagen in the supernatant was determined by hydroxyproline content and was adjusted to 1 mg/ml before i.v. injection. One group of rats was injected i.v. with 1 mg of 5× recrystallized ovalbumin (OVA) (Sigma Chemical Co., St. Louis, MO) that was dissolved directly into PBS (1 mg/ml). Intravenous injections were made via the lateral tail vein.

Immunization protocols. CIA was induced by immunizing rats with native BII that had been dissolved overnight at 4°C in 0.1 M acetic acid (4 mg/ml) and emulsified with an equal volume of IFA (Difco Laboratories, Detroit, MI). Rats were injected i.d. twice with 200 μg of emulsified BII. The first injection was made in a hind metatarsal footpad; the second, 7 days later, into the proximal one-third of the tail. In one study, rats were injected first with BII emulsified in complete Freund's adjuvant (CFA) (Difco Laboratories) and then reinjected with BII/IFA 7 days later. Immunization with BI or OVA in IFA was performed in an identical manner as BII.

Adjuvant arthritis was induced by injecting rats i.d. at the base of the tail with 250 μg of mycobacteria suspended in 0.05 ml of heavy mineral oil (E. R. Squibb and Sons, Princeton, NJ). Mycobacteria strains C.D.T. and P.N. were obtained from the Ministries of Agriculture, Fisheries and Food, Weybridge, Surrey, England, and were ground in an agate mortis and pestle with oil shortly before injection.

Determination of severity of arthritis. Rats were examined daily between days 10 and 28 for the presence and severity of disease. Afterward they were examined 2 or 3 times per wk for an additional 2 to 3 mo. Any rat developing CIA within the 4-mo duration of the study was included in calculations of incidence, onset, and severity of disease.

Severity of arthritis was determined subjectively by grading each limb on a scale of 0 to 4 as described (4). The maximum arthritis index (MAI) was calculated for each rat as the sum of the greatest score recorded for each limb. Thus, a score of 0 represents the absence of arthritis, and scores of 1 and 16 the mildest and worst disease, respectively. The MAI per group was calculated by the formula: number of arthritic rats × the mean MAI ÷ by number of rats in the group.

Collection of blood. Blood was obtained by venipuncture of the external jugular vein while rats were under light ether anesthesia. Blood was clotted at 4°C overnight and serum stored at −70°C until assay.

Antibody assay. IgG anti-collagen antibody was measured by an enzyme-linked immunosorbent assay (ELISA) system described by Rennard *et al.* (22) following minor modifications. Briefly, collagens or OVA were adsorbed to polystyrene microtiter plates (Nunc, Neptune, NJ) overnight at 4°C after being dissolved in 0.15 M potassium phosphate buffer, pH 7.6 (5 μg/ml). Plates were washed with 0.15 M NaCl (saline) containing 0.05% Tween 20. Samples of 100 μl of rat sera were added to duplicate wells after dilution in 0.1 M Tris-HCl buffered saline, pH 7.50, supplemented with 1% newborn calf serum and 0.5% Triton X-100. After a 1-hr incubation at 25°C, sera were removed, and the plates were washed. Peroxidase-conjugated goat anti-rat IgG (Cappel Laboratories, Cochranville, PA) was added at a predetermined dilution and incubated 90 min. After a final wash, 100 μl of orthophenylenediamine (OPD) substrate (40 mg OPD in 100 ml phosphate/citrate buffer, pH 5.0 and 40 μl of 30% H_2O_2) were added to each well. Colorimetric reactions of duplicate samples were read 1 hr later at 490 nm by using a Dynatech MR580 Microelisa Autoreader (Dynatech, Alexandria, VA) and were expressed as absorbance.

IgM anti-collagen antibody was measured by a modification of the procedure used to measure IgG. After rat sera were washed from the plates, rabbit anti-rat IgM (Miles Laboratories, Kankakee, IL) was added and incubated for 1 hr. The plates were then washed, and peroxidase-conjugated Fc-specific goat anti-rabbit IgG (Cappel Laboratories) was added as before. Ninety minutes later, the plates were washed and processed as described for IgG.

Serum dilutions of 1/100 and 1/1000, respectively, were used routinely to assay for IgM and IgG antibody in collagen-injected rats. Normal sera assayed at dilutions of 1/10–1/1000 yielded absorbance values of 0.003 to 0.012 for IgG and 0.040 to 0.050 for IgM. Sera from adjuvant-injected rats were assayed for anti-RII IgG at a dilution of 1/10.

This ELISA system is highly sensitive and able to detect affinity-purified rat anti-BII IgG at a concentration of < 10 ng/ml (absorbance 0.118); normal rat IgG (Cappel Laboratories) gave an absorbance reading of 0.013 when assayed at 10 ng/ml.

Measurement of delayed-type hypersensitivity (DTH). DTH was measured as the change in ear thickness (23) in millimeters (Δ mm) 48 hr after i.d. injection of 20 μg of BII dissolved in 0.02 ml of PBS. The opposite ear was injected with an equal volume of PBS and served as a control. Measurements were made with an engineer's micrometer and were expressed as the difference in thickness between collagen- and PBS-injected ears. Injection of BII into the ears of naive rats produced only negligible swelling (Δ 0.05 mm, i.e., 0.56 mm thickness preinjection; 0.61 mm 48 hr post-injection).

Measurement of BII in venous blood after i.v. injection. Clearance of immunoreactive BII from the circulation after i.v. injection was measured indirectly by ELISA by utilizing an inhibition assay. Rats were injected i.v. with 1 mg of BII, and blood was collected at regular intervals from the external jugular vein in heparinized syringes. A sample of 0.3 ml of whole blood was then mixed with 0.3 ml of diluted rat anti-BII serum. Equal amounts of blood from a noninjected donor or PBS served as controls. The mixtures of blood and antiserum were shaken gently on a platform rocker at 4°C for 1 hr. After centrifugation, 0.1-ml samples of the supernatants were added to microtiter plates coated with BII and were assayed as described. Sufficient amounts of blood were available from five bleedings to study the inhibitory activity of platelet-free plasma; normal plasma was used as a control in this instance. Inhibitory activity was not detected in whole blood or plasma obtained from normal rats. The percent inhibition was calculated by the formula:

$$\% \text{ Inhibition} = \frac{\text{Absorbance anti-BII serum + BII-injected rat blood or plasma}}{\text{Absorbance anti-BII serum + normal rat blood or plasma}} \times 100$$

Statistical analysis. Statistical significance of data was determined by using the χ^2 test with Yates correction or Student's *t*-test for nonpaired samples. Results are expressed as mean values ± standard error of the mean (SE).

RESULTS

CIA is specifically suppressed by i.v. injection of type II collagen. To determine whether the clinical course of CIA or the immune response to type II collagen could be modified, rats were injected i.v. with a single 1-mg dose of native BII or CII collagen dissolved in 1 ml of PBS (groups I and II). Control rats (groups III through IV, respectively) received 1 ml of PBS or 1 mg of OVA or BI dissolved in 1 ml of PBS. After a 7-day rest, all rats were challenged with BII/IFA. The results in Table I show that the incidence of arthritis was significantly reduced only in rats pretreated with BII or CII. In group I, only two of 11 rats developed CIA after challenge. One rat had mild, transient arthritis affecting a single hind limb (MAI, 3), and the other rat had severe arthritis involving 3 limbs (MAI, 11). None of the rats in group II given i.v. CII developed arthritis after challenge with BII/IFA. Six rats from group I that failed to develop CIA had their ankles examined histologically for evidence of subclinical arthritis and were found to be normal.

Rats in groups IV and V pretreated with antigens other than type II collagen were not protected, and arthritis developed in both groups at a similar incidence, severity, and day of onset as PBS controls (group III). These values for groups III–V are comparable to those previously reported in rats challenged with BII/IFA without i.v. pretreatment (3, 4, 9).

Anti-collagen antibody levels corresponded directly with the presence or absence of arthritis. IgM and IgG antibodies to BII and IgG cross-reactive with homologous RII were significantly depressed in rats pretreated with BII or CII (groups I and II). Suppression of anti-BII antibody was also examined by titering sera obtained 28 days after challenge from eight nonarthritic and eight arthritic rats (groups I and III, respectively). The rats in group I had a mean titer of 200 (range < 50 to 800) *vs* 51,200 (range 12,800 to 104,800) for arthritic rats in group III ($P < 0.001$) when titer was defined as the first serum dilution yielding an absorbance value of < 0.075. Finally, the mean anti-BII IgG value of nonarthritic rats from group I was compared with that of the five nonarthritic rats from groups III, IV, and V and was found to be significantly lower (0.047 ± 0.049 *vs* 0.581 ± 0.089, respectively, day 28, $P < 0.001$). The two rats in group I that developed arthritis after pretreatment had antibody levels that correlated with the severity of their arthritis (0.260 and 0.647). DTH responses measured by skin test reactivity to BII were also significantly depressed by i.v. pretreatment with BII (Table I).

Intravenous pretreatment with BII does not inhibit immunity to unrelated antigens. To determine if suppression of immunity to BII was specific and not due to an inhibitory or toxic property peculiar to type II collagen, six groups of rats were pretreated with BII or PBS before being sensitized with BI, OVA, or BII (Table II). The protocol for this study was identical to the one previously described except that rats in group X were challenged first with BII in CFA.

Pretreatment with BII suppressed immunity to only BII and

TABLE I

Suppression of CIA and immunity to type II collagen by i.v. injection of BII or CII[a]

	Group				
	I	II	III	IV	V
i.v. Pretreatment	BII	CII	PBS	OVA	BI
Incidence of CIA	2/11[b]	0/10[c]	8/10	9/10	8/10
X Day of onset	16.0 ± 2.0	0	13.4 ± 0.8	12.4 ± 0.3	12.3 ± 0.5
MAI					
Arthritic rats	7.0 ± 4.0	0	6.9 ± 0.9	8.4 ± 1.2	8.1 ± 0.5
Per group	1.3	0	5.5	7.6	6.5
IgM to BII day 16[d]	0.088 ± 0.022[c]	ND[e]	0.363 ± 0.026	0.487 ± 0.055	0.310 ± 0.042
IgG to BII day 16	0.028 ± 0.012[c]	ND	0.241 ± 0.026	0.374 ± 0.050	0.307 ± 0.070
IgG to BII day 28	0.190 ± 0.057[c]	0.162 ± 0.048[c]	0.710 ± 0.051	0.750 ± 0.07	0.660 ± 0.067
IgG to RII day 28	0.114 ± 0.059[c]	0.130 ± 0.045[c]	0.710 ± 0.050	0.748 ± 0.076	0.664 ± 0.067
DTH to BII day 30[f]	0.32 ± 0.060[c]	ND	1.15 ± 0.13	ND	ND

[a] Seven days before i.d. challenge with BII/IFA, rats were pretreated i.v. with 1 mg of BII, CII, OVA, or BI dissolved in 1 ml of PBS. Rats in group III received 1 ml of PBS i.v. Challenge is described in *Materials and Methods*.

[b] $P < 0.01$ *vs* groups III–V.

[c] $P < 0.001$ *vs* groups III–V.

[d] Antibody was measured by ELISA and expressed as absorbance. Values shown are the mean absorbances of arthritic and nonarthritic rats in each group ± SE. Antibody to BII was not detected in normal sera or the sera of six rats given i.v. BII 14 days earlier but not challenged with BII/IFA.

[e] Not done.

[f] DTH was measured by injecting on ear i.d. with 20 μg BII and the other with PBS. Values shown represent Δ in swelling in millimeters of study ear minus millimeters of control ear 48 hr with i.d. injection. DTH studies were performed on a separate group of rats (groups XV and XVIII, Table IV) pretreated i.v. with 1 mg of BII or 1 ml of PBS before challenge with BII/IFA. Only one of 10 BII-pretreated rats developed CIA *vs* eight of 10 controls.

TABLE II

Studies showing the specificity of suppression induced by BII[a]

Group	i.v. Pretreatment Day −7	Challenge Day 0	Challenge Day +7	Incidence of Arthritis	IgG Response to Respective Immunogen[b]
VI	BII	BI/IFA	BI/IFA	0/8	1.034 ± 0.35[c]
VII	PBS	BI/IFA	BI/IFA	0/8	0.972 ± 0.040
VIII	BII	OVA/IFA	OVA/IFA	0/6	0.425 ± 0.047[c]
IX	PBS	OVA/IFA	OVA/IFA	0/6	0.340 ± 0.036
X	BII	BII/CFA	BII/IFA	0/10	0.036 ± 0.008[d]
XI	PBS	BII/IFA	BII/IFA	8/10	0.753 ± 0.036[c]

[a] Rats were pretreated i.v. with 1 mg of BII or 1 ml of PBS 7 days before immunization with BI, OVA, or BII in IFA.

[b] Blood was collected 28 days after first challenge. Values are mean absorbance per group ± SE.

[c] Not significant *vs* PBS control.

[d] $P < 0.001$, group X *vs* XI.

[e] IgG cross-reactive to RII was 0.037 ± 0.008 and 0.750 ± 0.036 for groups X and XI, respectively.

TABLE III

Effect of time interval between i.v. injection of BII and challenge on the course of CIA[a]

Group	Injection (i.v.)	Day of i.v. Injection (Challenge Day 0)	Incidence of Arthritis	IgG Response[b] Day +28
XI	BII	−32	1/5	ND
	PBS		4/6	ND
XII	BII	−7	2/11[c]	0.190 ± 0.057[d]
	PBS		8/10	0.710 ± 0.051
XIII	BII	−4	1/10[c]	0.040 ± 0.012[d]
	PBS		8/10	0.717 ± 0.064
XIV	BII	+7	1/10[c]	0.264 ± 0.061[d]
	BI		7/10	0.850 ± 0.120

[a] Rats were injected i.v. with 1 mg of BII before or after challenge as shown. Control rats received an equal 1-ml volume of PBS at the same time, except for group XIV, which was injected i.v. at day +7 with 1 mg of BI solubilized in PBS. Challenge was performed as described earlier. ND, not done.

[b] Mean absorbance ± SE.

[c] $P < 0.01$ *vs* PBS control.

[d] $P < 0.001$ *vs* PBS- or BI-injected controls.

had no effect on IgG responses to either BI or OVA. Moreover, the suppression induced by i.v. pretreatment with BII could not be overcome by using CFA, a more potent immunoadjuvant.

BI was found to be a potent immunogen producing absorbance values greater than BII. This finding differs from an earlier report in which heterologous chick type I collagen was described as a poor immunogen in the rat when emulsified with IFA (2). The discrepancy between that study and ours may reflect either differences in the sensitivity of hemagglutination and ELISA assays for antibody to type I collagen or, alternatively, differences in the immunogenicity of these type I collagens.

Importance of time interval between i.v. injection of BII and i.d. challenge. The incidence of different injection schedules on the course of CIA and immunity to BII is shown in Table III. The incidence of CIA was reduced significantly when 1 mg of BII was given i.v. 32, 7, or 4 days before challenge or 7 days after. Anti-BII IgG was suppressed in all groups given BII as compared with controls given PBS ($P < 0.001$). Arthritis and antibody to BII were observed in a few rats (5 of 36) given i.v. BII before or after challenge, however, the day of onset, severity, and IgG values were variable. In subsequent studies suppression of CIA and antibody to BII has been virtually 100% effective suggesting that occasional failures may have resulted from technical problems. (Data not shown).

The course of rats given i.v. BII 7 days after challenge (group XIV) was different from those pretreated with BII. Nine of 10 rats in group XIV became acutely ill within 24 hr of i.v. injection, developed proteinuria, and lost weight. Clinical changes and proteinuria were not seen in 10 control rats given BI at the same dose and time. The kidneys of one rat examined histologically 7 days after the onset of proteinuria disclosed a mild glomerulonephritis suggesting injury by BII-immunoglobulin complexes. Immunofluorescence studies, however, were not performed. Clinical changes and proteinuria were not found in a group of six rats given i.v. BII alone or 10 rats given i.v. BII and then challenged 7 days later. (Data not shown). Histologic examination of the kidneys of six untreated arthritic rats disclosed no abnormality.

Finding that arthritis could be suppressed after challenge, we attempted to inhibit active arthritis. A group of six rats with severe arthritis, immunized 32 days earlier with BII/IFA, was chosen for study. After an initial bleeding, rats were rested for 2 days and then given 1 mg of BII i.v.; blood was collected 2 and 21 days later. No detectable change in the severity of arthritis was noted after collagen injection. Anti-BII IgG values for the three bleedings were 0.810 ± 0.089, 0.512 ± 0.106, and 0.813 ± 0.137, respectively, indicating only a slight, possibly transient fall in IgG.

Proteinuria was not detected in rats given BII i.v. 32 days after immunization. This finding suggests that the ratio of antigen and antibody may determine the pathogenicity of BII-immunoglobulin complexes formed *in vivo*.

Importance of dose and route of injection of BII. Suppression

of CIA was dependent on the amount of BII given and the route of injection as shown in Table IV. Pretreatment was performed as before except i.v. injections were given 4 days before challenge. This was done for convenience because 4- and 7-day pretreatment schedules were quite effective in suppressing arthritis and immunity to BII.

Only one of 10 rats given 1 mg of BII i.v. developed CIA in contrast to eight of 10 controls pretreated with 1 ml of PBS ($P < 0.01$). Rats pretreated with 0.1 or 0.01 mg of BII in equal volumes of PBS developed arthritis at an incidence and severity comparable to controls though at later dates of onset. Rats pretreated with 1 mg of BII s.c. also developed arthritis similar to controls.

Sera of nonarthritic rats, pretreated i.v. with 1 mg of BII, contained only traces of IgG specific to BII as compared with PBS-injected controls. The one arthritic rat in this group developed an IgG value of 0.677, which was comparable with arthritic controls. Sera of rats pretreated with 0.1 or 0.01 mg of BII contained intermediate amounts of IgG suggesting a slight inhibition of response consistent with the delayed onset of arthritis.

Adjuvant arthritis is not suppressed by pretreatment with BII. To determine if AIA could be suppressed by i.v. pretreatment with BII and if rats with AIA produce antibody to RII, an additional study was done. Adjuvant arthritis was induced in three groups of rats by a single i.d. injection of mycobacteria suspended in oil. Groups one and two each contained 10 rats and received i.v. pretreatment with 1 mg of BII or 1 ml of PBS 4 days before i.d. injection with adjuvant. Heterologous BII was used instead of homologous RII because of BII's strong cross-reactivity with RII and ability to suppress immunity to RII in rats immunized with BII/IFA and BII/CFA (Tables I and II). Moreover, in preliminary studies, BII was found to be more effective than RII in inducing and suppressing arthritis (unpublished observations). Group three contained 26 rats and received no i.v. pretreatment before injection with adjuvant. The latter group was bled weekly from day 14 through day 35 to provide sera for antibody studies. Severe arthritis developed in all 46 rats.

Pretreatment with BII affected neither the incidence, severity, time of onset of AIA, nor the incidence of ear nodules or spondylitis, compared with rats pretreated with PBS (Table V). Furthermore, anti-RII IgG was not detected at a 1/10 dilution in any of the sera assayed by a sensitive ELISA system that readily detected antibody in rats with CIA.

Clearance of immunoreactive BII from peripheral blood. The rate solubilized BII was cleared from the circulation after i.v. injection was indirectly determined by ELISA utilizing an antibody inhibition assay. Two normal rats were given an i.v. injection of 1 mg of BII, and venous blood was collected in heparinized syringes at regular intervals.

The upper curve in Figure 1 shows the clearance of BII from whole blood. Two distinct slopes are apparent, an early one lasting between 15 and 60 min, which shows rapid clearance; and a later one lasting between 2 and 8 hr, which shows a more gradual clearance of BII. The lower curve shows the clearance of BII from platelet-free plasma, which was complete within 2 hr. These data imply that BII present 2 hr after i.v. injection was bound to cellular elements. In earlier studies, performed *in vitro*, we found that BII spontaneously bound to splenocytes and erythrocytes (unpublished observation) suggesting that a similar process may occur *in vivo* after i.v. injection.

DISCUSSION

In this report, we have shown that soluble native type II collagen induces and suppresses CIA in rats depending on the mode of its administration. Rats immunized i.d. with BII in IFA developed arthritis at an incidence of 70 to 90% and developed a strong immune response to type II collagen. When immunization was preceded or followed shortly by an i.v. injection of soluble type II collagen, however, the incidence of arthritis was reduced to 0 to 18%, and immunity to collagen was significantly suppressed.

Our data suggest that CIA was prevented by the induction of immune tolerance. The suppression of immunity was antigen specific, dependent on the i.v. route of injection, and related to the dose of BII. The antigen-specific nature of suppression was shown in two studies. First, neither arthritis nor immunity to BII were suppressed by PBS, BI, or OVA given i.v. before challenge with BII/IFA. Conversely, it was also found that i.v. pretreatment with BII had no effect on immunity to BI or OVA when rats were immunized with BI/IFA or OVA/IFA. Both studies demonstrate a) that the suppression of CIA, like its induction, is critically dependent on collagen type, and b) that nonspecific mechanisms of suppression, i.e., stress (17), and the activation of antigen-independent suppressor T-cells (24) or macrophages (25), were not triggered by i.v. injected collagen. Lastly, we found that suppression of CIA was not dependent on the species source of type II collagen used for pretreatment. Intravenous injection of BII or CII was effective in suppressing arthritis and anti-RII IgG in rats challenged with BII/IFA. This data suggests that bovine- and avian-derived type II collagens share significant antigenic homology with homologous RII.

On comparing our results with previous models of tolerance, the rapid clearing of BII from the blood after i.v. injection is in sharp contrast with reports that other soluble proteins used as tolerogens circulate for weeks after i.v. injection (26). The rapid clearance of BII demonstrates that collagen need not circulate for long periods to induce or maintain suppression.

Our studies on inducing tolerance after immunization are consistent with the work of other investigators who examined different antigens. We found, as did Sanfilippo and Scott (26), that immunity could be suppressed in an antigen-specific manner when antigen was administered i.v. 7 days after immunization. No suppression of arthritis or immunity to BII, however, was seen when i.v. injection was delayed 32 days after immunization.

TABLE IV

Dose response of BII, route of injection, and the course of CIA[a]

Group	Dose of BII per Rat	Route	Incidence of Arthritis	MAI		Mean Day of Onset ± SE	IgG Response at Day 28 ± SE
				Arthritic rats	Per group		
XV	1.0 mg	i.v.	1/10[b]	3	0.3	35	0.040 ± 0.012[b]
XVI	0.1 mg	i.v.	7/10	6.0 ± 0.7	4.2	25 ± 2.8[c]	0.412 ± 0.070
XVII	0.01 mg	i.v.	9/10	5.4 ± 1.4	4.7	20.2 ± 1.3[c]	0.499 ± 0.107
XVIII	PBS	i.v.	8/10	6.8 ± 0.8	5.4	13.1 ± 1.0	0.717 ± 0.064
XIX	1.0 mg	s.c.	6/10	6.2 ± 0.9	3.7	18.7 ± 4.5	ND

[a] Rats were injected i.v. with 1.0, 0.1, or 0.01 mg of BII solubilized in 1 ml of PBS. Controls in group XVIII received 1 ml PBS. Rats were challenged 4 days later as described. ND, not done.

[b] $P < 0.01$ *vs* group XVIII.

[c] $P < 0.001$ *vs* group XVIII.

TABLE V

Pretreatment with BII does not suppress adjuvant arthritis

	Intravenous Pretreatment[a]		
	BII	PBS	None
Incidence of AIA	10/10	10/10	26/26
MAI	14.7 ± 0.6	15.0 ± 0.4	12.4 ± 0.6
Arthritic limbs/group	39/40	40/40	95/104
Mean day of onset	11.9 ± 0.5	11.1 ± 0.3	11.9 ± 0.2
Incidence of:			
Ear nodules	10/10	10/10	26/26
Spondylitis[b]	9/10	10/10	NR[c]

[a] Rats in each group received i.v. pretreatment with 1 mg of BII or 1 ml of PBS 4 days before I.d. injection with 250 μg of ground mycobacteria suspended in oil.

[b] Spondylitis was defined as kyphotic deformity of the spine or ankylosis of the tail distal to the site of adjuvant injection.

[c] NR, not recorded.

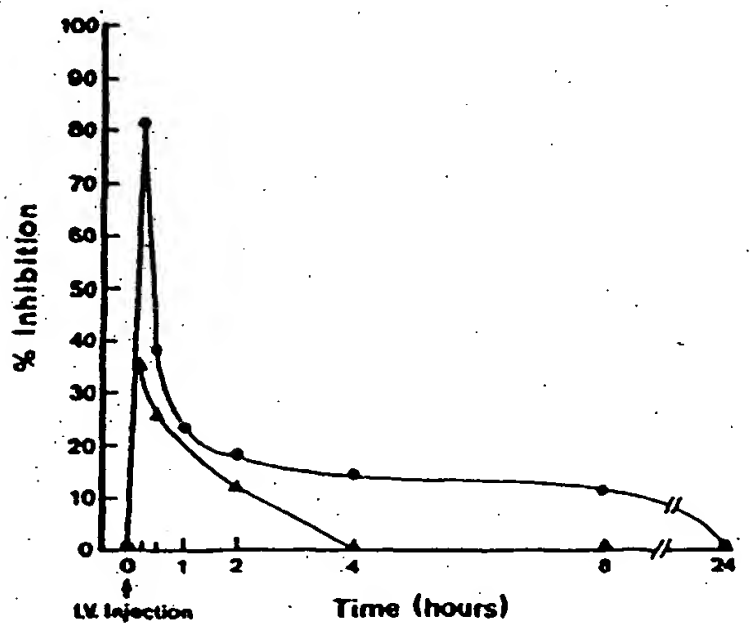

Figure 1. Clearance of immunoreactive BII from peripheral venous blood of a normal rat injected I.v. with 1 mg solubilized BII. Assay for BII was performed by measuring the inhibitory activity of circulating BII against anti-BII IgG in an ELISA system. ●, represent the inhibitory activity of whole blood; ▲, the inhibitory activity of platelet-free plasma.

Other workers have also reported difficulty in inducing tolerance 2 to 4 wk after immunization (27, 28).

Suppression of CIA with type II collagen has been reported by two other groups. Schoen *et al.* (14) found that the incidence of CIA in rats could be reduced, in an antigen-specific manner, by i.v. injection of CII-coupled spleen cells. A series of four injections reduced anti-CII IgG levels by ≈50% as measured by radioimmunoassay. This treatment, however, had no effect on hemagglutination titers or DTH reactivity to CII. In comparison, immunosuppression induced by i.v. injection of soluble collagen, which suppressed antibody and DTH, was greater than that reported by Schoen and co-workers. Differences between their study and ours may be attributable to the form or amounts of collagen injected.

Staines *et al.* (18) recently found that CIA and anti-RII IgG were suppressed by treating rats with i.v. injections of pig type II collagen (100 μg/rat) and immune serum (1 ml/rat) given 8 and 9 days before i.d. challenge, respectively. Pretreatment with only collagen or serum produced a modest suppressive effect, whereas the combination was quite effective. Our study also showed a modest degree of antibody suppression when 100 μg of type II collagen were used for pretreatment. Although these investigators did not study the effects of larger doses of collagen, our work shows that at least 1 mg of type II collagen is needed to produce significant suppression.

The importance of immunity to type II collagen in the pathogenesis of CIA has been amply demonstrated (1–21). Work from this laboratory (2, 9) and others (6, 7) has shown that immunizing rats with homologous or heterologous type II collagens, prepared from a variety of species, induces arthritis and immunity to homologous RII. The critical importance of antibody to type II collagen in the development of CIA is now clearly established. Morgan *et al.* (12) first suggested this by showing that arthritis did not appear in BII-immunized rats as long as serum C was depleted. This finding and the work of Schoen *et al.* (14) indirectly emphasized the importance of humoral immunity in CIA and questioned the role of cell-mediated immunity (CMI) to collagen in CIA. Recently, studies from this laboratory provided direct evidence for an antibody-mediated pathogenesis of CIA by showing that IgG specific for type II collagen produces arthritis when passively transferred to normal recipients (15, 16).

Several investigators have suggested that autoimmunity to collagen might play a role in the pathogenesis of AIA (29–33). Steffen and co-workers were the first to report that adjuvant arthritic rats developed weak DTH to denatured (30) and native (31) type I collagen. Trentham *et al.* (32) reported CMI to native and denatured homologous type I and II collagens in rats with AIA. Recently, Holoschitz *et al.* (33) isolated an effector T cell line from adjuvant arthritic rats that proliferated strongly to mycobacteria and weakly to RII. They found this cell line effective in transferring or vaccinating against AIA. A T cell line reactive only to RII from rats immunized with RII/IFA was ineffective in transferring CIA. Furthermore, Schoen *et al.* (34) showed that s.c. injection of RII-coupled spleen cells induced RII-specific CMI in rats but neither arthritis nor antibody to RII. These findings and those previously reported by others (12, 14) suggest that CMI to type II collagen alone is not sufficient to induce arthritis. These data, however, do not exclude the importance of Th cells in the genesis of immunity to type II collagen, a T-dependent antigen, nor the possibility that CMI may contribute to tissue injury once arthritis is initiated (35).

Antibodies to native and denatured type I and II collagens, measured by hemagglutination, have been reported in ≈40% of rats with AIA (32). The authors of that report, however, were unable to conclude whether anti-collagen antibodies represented a primary or secondary event. In contrast to CIA, in which antibodies levels are high, appear before the onset of arthritis, and react primarily to type II collagen, antibody titers described in AIA were frequently low, appeared after the onset of arthritis, and reacted well to type I and II collagens. These data suggest to us that immunity to collagen in adjuvant disease represents a secondary response produced by injury of articular tissues or granuloma formation. In our studies, we were not able to detect antibody to type II collagen in severe AIA by using a sensitive ELISA system.

Other reports provide data suggesting that CIA and AIA have dissimilar primary pathogenetic mechanisms. We first suggested this possibility when we found that type II collagen lacked adjuvant activity (36), a property intimately associated with the ability of natural (37) and synthetic substances to induce adjuvant arthritis (38, 39). Our work has been since confirmed by Iizuka and Chang (40). Furthermore, these investigators showed that AIA could be suppressed by pretreatment with a subarthritogenic dose of mycobacteria given s.c. before immunization with adjuvant, whereas the same procedure had no effect on CIA. Data presented here show, conversely, that i.v. pretreatment with BII suppressed CIA and immunity to BII and RII but had no suppressive effect on AIA. This observation and the absence of anti-RII IgG in adjuvant arthritis further support the conclusion that immunity to type II collagen is not central to the pathogenesis of AIA.

In conclusion, studies reported here show that CIA in rats and

immunity to type II collagen can be suppressed in an antigen-specific manner by I.v. injection of native type II collagen. This data further supports the hypothesis that CIA is mediated by immunity to type II collagen. Furthermore, attempts to suppress AIA by I.v. injection of collagen and to demonstrate anti-collagen antibody in AIA were not successful. These findings suggest that CIA and AIA, despite some phenotypic similarities, are distinct entities mediated by different primary pathogenic mechanisms.

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Control of Cellular and Humoral Immune Responses by Peptides Containing T-cell Epitopes

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T cells are known to recognize antigen in the form of peptides bound to major histocompatibility complex (MHC)-encoded class II molecules (Babbit et al. 1985; Schwartz et al. 1985; Buus et al. 1987; Guillet et al. 1987). Experiments with MHC-encoded class II molecules in planar membranes show that the binding of the T-cell receptor (TCR) to the peptide/MHC complex may not be sufficient for activation of the responding T cells (Quill and Schwartz 1987). One signal to the T cells is the binding of the TCR/CD3 complex to the antigen/MHC. As proposed by Bretscher and Cohn (1970), lymphocytes may be tolerized unless they see a second signal. This second signal for T cells can be provided in principle by the antigen-presenting cell (APC), either through the secretion of soluble cytokines or by the interaction of molecules on the APC and T-cell surface. To test this hypothesis, we exposed mice to peptides containing known T-cell epitopes without adjuvant in order to expose the T cells only to the first (TCR) signal.

The biological relevance of the existence of various APC types such as macrophages, B cells, and dendritic cells in vivo is still unclear. As mentioned above, the second signal necessary for the activation of T cells may be provided by APCs (Quill and Schwartz 1987). It is also possible that different types of APCs may provide different second signals and may thus affect the quantitative and qualitative outcome of immune responses. In this paper, the responding T cells from mice immunized with the amino-terminal fragment of λ repressor cI 1-102 using different immunization protocols are characterized with respect to the distribution of T-cell epitopes recognized, as well as their functional phenotypes upon presentation of T-cell epitopes by different APCs in vitro.

The protein context of a peptidic T-cell epitope also influences the T-cell response specific for that epitope (Shastri et al. 1986). Thus, the immune response to a T-cell epitope may be hidden when it is covalently linked to another T-cell epitope. This can be due, for example, to competition between epitopes for binding to the MHC peptide-binding site (Guillet et al. 1987). Antigen processing can also explain differential expression of epitopes, depending on the context. Thus, neighboring or distal residues could influence processing so that a particular T-cell epitope is unavailable to bind MHC. We compare the activity of an ovalbumin-derived peptide epitope that is suppressed when covalently linked to an epitope of greater immunogenicity in an antigenic and tolerogenic system.

MATERIALS AND METHODS

Animals. Strains BALB/cByJ, C57BL/6 mice were obtained from Jackson Laboratories, Bar Harbor, Maine.

Cell cultures and assay conditions. All cultures and assays were performed according to established procedures (Lai et al. 1987).

Antigens. Bacteriophage λ repressor cI protein fragment p1-102 and synthetic peptides were prepared as described previously (Lai et al. 1987). p12-26^{N14} is residues 12–26 of cI with asparagine substituted for aspartic acid at position 14.

Cell lines. BW5147.G.4.Oau^{R}.1(α-,β-) was a gift from W. Borne. A20.2J (I-A^{d}, I-E^{d}) was a gift from J. Kappler and P. Marrack. CTLL.2 was a gift from D. Raulet. CT.4S (Hu-Li et al. 1989) and 11B11 (anti-IL-4) (Ohara and Paul 1985) were a gift from W. Paul. S4.B6 (anti-IL-2) was a gift from T. Mosmann. Class II MHC L-cell transfectants RT 2.3.3H (I-A^{d}) and RT 10.3H2 (I-E^{d}) were a gift from R. Germain.

Antibodies. Monoclonal antibodies were produced by harvesting cell supernatants from B-cell hybridomas. Supernatants were centrifuged and sterile filtered to remove any residual cells. 11B11 supernatant was used at 10%, and S4B6 supernatant was used at 50%.

Antisera. BALB/c mice were tolerized by injection of p12-26 i.v. in saline, or saline alone as a control. They were immunized 10 days after the first tolerization with p12-26 i.p. (50 μg emulsified in complete Freund's adjuvant [CFA]). Mice received a booster immunization 14 days after the primary injection of p12-26 i.p. (50 μg emulsified in incomplete Freund's adjuvant [IFA]). Mice were bled 7 days after the first immunization and 8 days after the booster immunization (data shown). Antibodies were measured by enzyme-linked immunosorbent assay (ELISA) (Good et al. 1988).

Isolation of T-cell hybridomas. Mice were immunized either s.c. at the base of the tail and in both thighs or i.p. with 100 μg of bacteriophage λ repressor p1-102 in either CFA, IFA, or alum. After 7 days, draining lymph nodes were removed from mice with s.c. immunizations, and spleen cells were removed from those with i.p. immunizations. Cells were stimulated in vitro with p1-102 at 50 μg/ml for 2 days before fusion with BW5147. Fusion hybrids were prepared according to established procedures (Gefter et al. 1977).

All the T-cell hybridomas reported here have been subcloned at least once by limiting dilution method (Walker et al. 1982). 9C127 and 1E1 are hybridomas made from BALB/c mice. 3D054.8 is a clone specific for OVA(325-336).

Tolerization of adult mice. Mice 4–8 weeks old were injected i.v. in the tail vein with 300 μg of deaggregated peptide dissolved in 100 μl of saline on day 0. After 5 days, another 300 μg of peptide was injected i.v. On day 10, the mice were immunized s.c. with peptide in CFA as described above.

Lymphokine assays. IL-2 assays were performed as described previously (Lai et al. 1987). Assay for IL-4 was performed using the IL-4-dependent cell line CT.4S according to the method of Hu-Li et al. (1989), with the slight modification that tritium incorporation in DNA was measured 6 hours after the addition of 1 mCi of tritiated thymidine.

Lymphocyte proliferation assays. T-lymphocyte proliferation assays were performed according to methods described by Lai et al. (1987).

RESULTS

We have previously shown that the T-cell response in BALB/c mice to the amino-terminal fragment of λ repressor (p1-102) is predominantly directed to a synthetic peptide (p12-26) containing residues 12–26 (Guillet et al. 1987; Roy et al. 1989). Proliferation of p1-102-immunized T cells is equivalent in response to either p1-102 or p12-26, and over 90% of BALB/c-derived T-cell hybrids made against p1-102 respond to p12-26. In addition, p12-26 can serve to recruit help, since immunization of mice with p12-26 produces anti-p12-26 antibodies (Roy et al. 1989). When BALB/c mice are exposed to p12-26 by intravenous injection, they become unresponsive to later immunization with p12-26 s.c. in CFA. The same is true for p12-26^{N14}, a related peptide that cross-reacts with p12-26 (Fig. 1). The tolerization procedure decreases lymphocyte proliferation (Fig. 1a) and almost eliminates IL-2 secretion (Fig. 1b) of draining lymph node cells. The effect is epitope-specific, since i.v. injection of saline alone or of unrelated peptides has no effect. In addition, C57BL/6 mice can be tolerized to their immunodominant epitope in p1-102, p73-88 (data not shown). This tolerization protocol also eliminates T-cell help, since treated mice can no longer produce antibodies to p12-26 (Table 1).

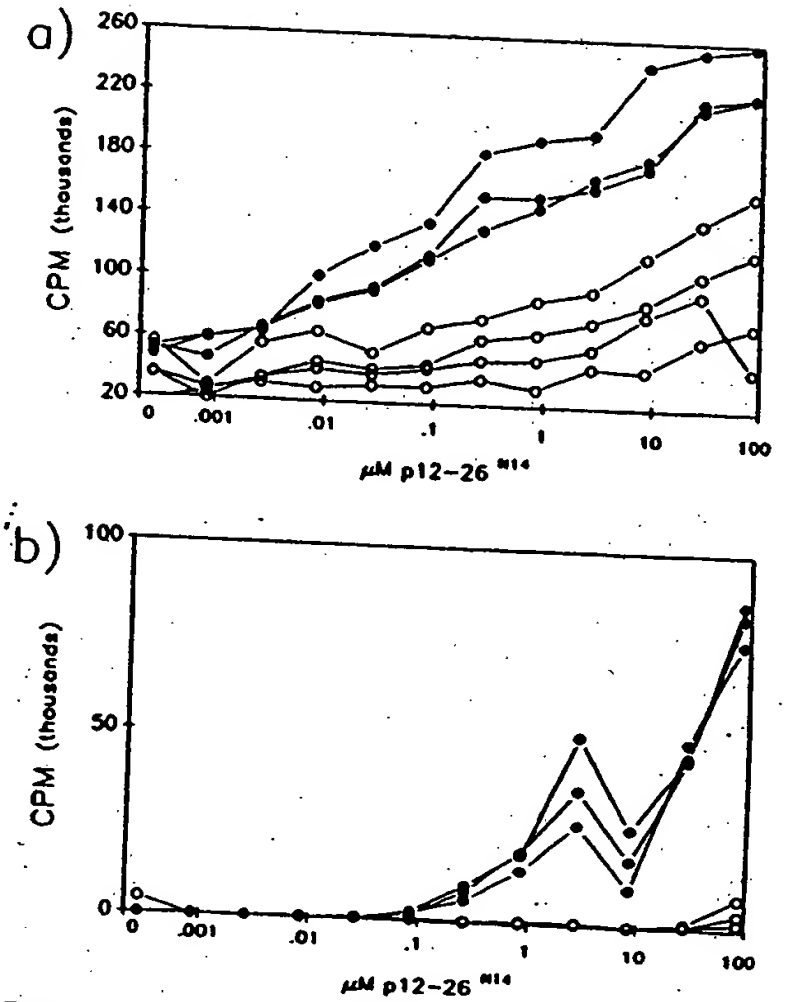

Figure 1. Response of lymphocytes from BALB/c mice immunized with p12-26^{N14} s.c. (○) Mice tolerized by two i.v. injections of p12-26^{N14}; (●) control mice. (*a*) Proliferation; (*b*) IL-2 secretion as measured by proliferation of CTLL cells.

The reduced response cannot be due to a sudden high concentration of soluble antigen inactivating T cells, since the same tolerization effect can be seen when antigen is injected i.p. in IFA 10 days before immunization (data not shown).

The tolerance effect lasts for at least 6 weeks (Table 2). The T-cell proliferation is initially reduced to only 20% of that of immunized mice, whereas the IL-2 secretion is reduced even more, to less than 5%. After 42 days, the IL-2 secretion of tolerized T cells seems to have increased to 15% of that of control T cells, whereas only one of two mice tested have reduced proliferation. Studies are continuing to investigate further the duration of the T-cell nonresponsiveness.

The T-cell tolerance could be due to the inactivation of antigen-specific T cells or the induction of suppressor

Table 1. Antibody Response to p12–26

Mouse	Pre-bleed (μg/ml)	Immunized (μg/ml)
Tolerized	<2	19
Tolerized	3	12
Tolerized	3	15
Tolerized	3	5
Control	2	528
Control	4	378
Control	2	99
Control	3	363

Table 2. Persistence of Tolerization Effects

Day immunized	% of Control	
	IL-2	proliferation
1	3, 2	19, 19
5	1, 2	28, 38
9	6, 20	14, 39
41	11, 17	17, 103

BALB/c mice were i.v. tolerized on day −5 and day 0 p12-26. Mice were immunized at various times thereafter and tested for specific proliferation and IL-2 secretion 7 days later.

T cells. To test the latter possibility, mixtures of lymphocytes from tolerized and nontolerized mice were compared with mixtures of lymphocytes from nonimmunized and nontolerized mice. It is expected that if tolerization were due to the induction of suppressor T cells, then the suppressor T cells present in tolerized mice should suppress the response of immunized T cells, whereas mixing with nonimmunized T cells should have no effect. As seen in Figure 2, the proliferation of immunized cells mixed with tolerized cells was the same as that of immunized cells mixed with nonimmune cells. If anything, the response was a bit higher, as could be expected, because tolerized cells still proliferate at 20% of the control level. Furthermore, the proliferation clearly rises as more immunized cells are added, until, with 99% control immunized cells and only 1% tolerized or nonimmunized cells, the response is normal. The same result was obtained with IL-2 secretion (data not shown).

We then investigated the immune response to a molecule in which two T-cell epitopes were combined. The two epitopes chosen were residues 12–26 (p12-26) of λ repressor and residues 325–336 (OVA-D) of egg ovalbumin, with valine residue 327 changed to aspartic acid to reduce binding to I-A^d (Sette et al. 1987). Both epitopes are I-A^d-restricted, and p12-26 and ova325-336 are immunodominant in their respective proteins when injected into BALB/c mice (Guillet et al. 1987; Shimonkevitz et al. 1987). We synthesized a long joint peptide, 12-26-GPG-OVA-D, containing p12-26 linked to OVA-D by a glycine-proline-glycine bridge (see Table 3). T cells from BALB/c mice immunized with OVA-D proliferate and secrete IL-2 when stimulated in vitro with OVA-D. Similarly, T cells from mice immunized with p12-26 proliferate and secrete IL-2 in response to either p12-26 or the joint peptide (Fig. 3a). In contrast, lymphocytes from mice immunized with the long peptide respond in vitro to the long peptide and to p12-26, but not to OVA-D (Fig. 3e). Thus, the OVA-D epitope is hidden when present within the joint peptide. The basis for this result could be easily explained by competition between the two epitopes for binding to MHC-encoded class II molecules and perhaps also to a third epitope created at the junction of the two fused epitopes. This epitope suppression is not due to the inability of BALB/c-presenting cells to present OVA-D from within the long peptide, since the long peptide stimulates the ova325-336-restricted T-cell hybridoma 3DO54.8 when presented on I-A^d-transfected L cells (Fig. 4a). I-A^d-transfected L cells (Fig. 4b) and fixed or live H-2^d A20 cells (Fig. 4c) can also present the joint peptide to p12-26-specific hybridomas 9C127(I-A^d-restricted) and 1E1 (I-E^d-restricted). Thus, processing of the joint peptide is not required, at least for presentation of the p12-26 epitope in vitro.

Mice immunized with p12-26, which 10 days earlier had been tolerized by i.p. injection with either p12-26 (Fig. 3b) or the joint peptide (Fig. 3d) in IFA, have diminished responses, whereas mice given saline or OVA-D-tolerized mice proliferate normally in response to either p12-26 or the joint peptide (Fig. 3a,c). Similarly, mice immunized with the joint peptide that were previously tolerized with the joint peptide cannot respond to either p12-26 or the joint peptide (Fig. 3h), and those tolerized to p12-26 cannot respond to p12-26 (Fig. 3f) but can respond to a lesser extent to the joint peptide, possibly because T cells specific for the junction were not tolerized. Control mice tolerized to OVA-D (Fig. 3g) or not tolerized (Fig. 3e) respond to both p12-26 and the joint peptide. Mice immunized with OVA-D that were previously tolerized with OVA-D cannot respond to OVA-D or the joint peptide (Fig.

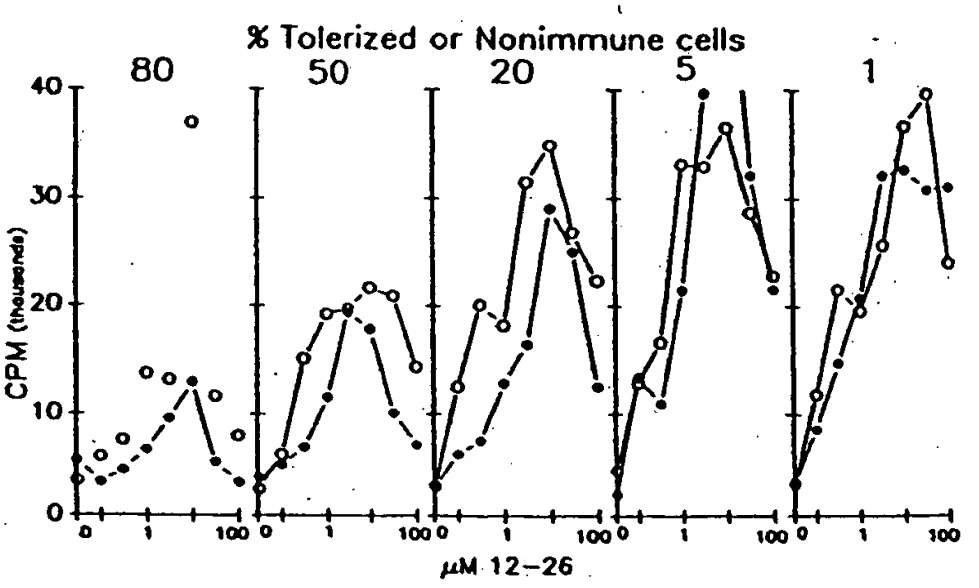

Figure 2. Proliferation of lymphocytes from mice immunized with p12-26. Lymphocytes from control mice were mixed with various percentages of lymphocytes from either tolerized mice (O) or nonimmunized mice (●).

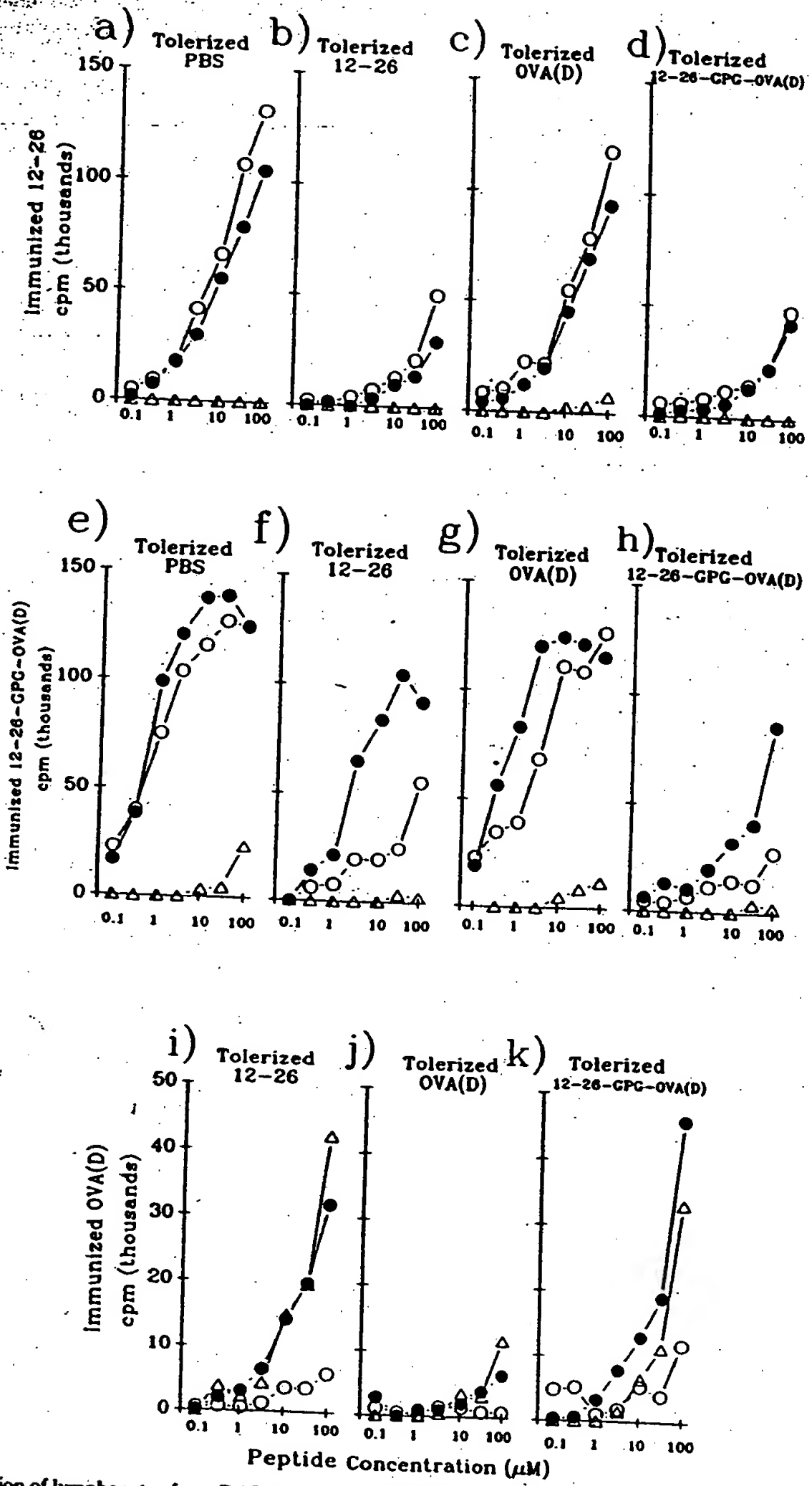

Figure 3. IL-2 secretion of lymphocytes from BALB/c mice. Lymphocyte response to in vitro stimulation with (●) joint peptide, (○) p12-26, (△) OVA-D. (*a*) Mice sham-tolerized and immunized with p12-26; (*b*) mice tolerized with p12-26 and immunized with p12-26; (*c*) mice tolerized with OVA-D and immunized with p12-26; (*d*) mice tolerized with joint peptide and immunized with p12-26; (*e*) mice sham-tolerized and immunized with joint peptide; (*f*) mice tolerized with p12-26 and immunized with joint peptide; (*g*) mice tolerized with OVA-D and immunized with joint peptide; (*h*) mice tolerized with joint peptide and immunized with joint peptide; (*i*) mice tolerized with p12-26 and immunized with OVA-D; (*j*) mice tolerized with OVA-D and immunized with OVA-D; (*k*) mice tolerized with joint peptide and immunized with OVA-D.

Table 3. Sequences of Peptides Used

P12–26	LEDARRLKAIYEKKK
P12–26^{N14}	LENARRLKAIYEKKK
OVA-D	QADHAAHAEINE
Joint peptide	LEDARRLKAIYEKKKGPGQADHAAHAEINE

3j), but mice that were tolerized with p12-26 do respond (Fig. 3i). However, mice tolerized with the joint peptide and immunized with OVA-D still respond to both OVA-D and the joint peptide (Fig. 3k). Thus, the OVA-D epitope is hidden within the joint peptide during tolerization as well as immunization. It appears that tolerization to T-cell epitopes occurs when such epitopes are presented by APCs but in the absence of a "second signal."

The mode of immunization is known to influence the immune response (Warren et al. 1986). For the purposes of this study, we may ask what types of second signals promote what types of immune responses. T-cell hybridomas were prepared from BALB/c mice immunized with p1-102 in CFA s.c., CFA i.p., IFA s.c., IFA i.p., and alum i.p. They were originally screened by measuring IL-2 secretion, as tested for by the ability to sustain growth of the IL-2-dependent cell line CTLL. Previous studies (Roy et al. 1989) have shown that p12-26 is the immunodominant epitope in BALB/c mice immunized with p1-102 in CFA s.c.. As shown in Table 4, p12-26 was also immunodominant in immunized mice using the five different protocols of immunization. About 90% of all p1-102-specific hybrids respond to p12-26. In addition, about half of the non-p12-26 hybrids were found to respond to a second p1-102 peptide, p46-62.

The p46-62- and p12-26-specific hybrids were further characterized with respect to their production of IL-4, using the IL-4-dependent clone CT.4S as indicator cells. Interestingly, over 50% of the p46-62-specific hybridomas produced IL-4 upon stimulation by B cells (A20) as APCs (Table 5). In contrast, less than 5% of the p12-26-specific hybridomas produced IL-4. A representative p12-26-specific hybridoma, 1PA12-1, produced IL-2 when given p12-26 presented on either the B cell A20 or I-A^d-transfected L cells (Fig. 5a,b). The CTLL response was inhibitable by anti-IL-2 antibody. In contrast, there was no response when the supernatants were tested on CT.4S cells (Fig. 5c,d). Thus, T cells that recognize p46-62 often secrete IL-4, whereas those that recognize p12-26 rarely do. It is possible that this observation is due to presentation by distinct subsets of APCs that may direct responding T cells to express different functional phenotypes. We propose that APCs that can process p1-102 to give rise to p46-62 leading to IL-4 production are a subset of APCs distinct from those that process p1-102 primarily to p12-26. Evidence supportive of this possibility was obtained by comparing the ability of B cells (A20) and I-A^d-transfected L cells to stimulate IL-4 production from the identified hybridomas. A representative p46-62-specific hybridoma, 1SI46l-1.1, produces IL-2 when A20 cells are used as APCs, as shown by a CTLL response that is inhibitable by anti-IL-2 and not by anti-IL-4 (Fig. 6a). IL-4 production was also observed by stimulation of CT.4S, which is inhibited by anti-IL-4 and not by anti-IL-2 (Fig. 6c). The small decrease in the CT.4S response in the presence of anti-IL-2 may be due to a synergistic effect of IL-2 on IL-4 secretion on CT.4S stimulation (Hu-Li et al. 1989), as well as to the nonspecific toxicity of the antibody culture supernatant used. When I-A^d-transfected L cells are used to present p46-62, production of IL-2 but not IL-4 is observed (Fig. 6b,d). No hybrid tested has been able to secrete significant amounts of IL-4 when I-A^d-transfected L cells were used as APC. This is not due simply to a dose-response shift of the L cells as compared to A20 cells. The dose response of using L cells shifts 3-fold for the IL-2 response (Fig. 6a,b), whereas there is no IL-4 response while using L cells even at 32 μM of p46-62, 32-fold more antigen than required with A20 cells (Fig. 6c,d).

DISCUSSION

One known mechanism of thymic tolerance depends on the deletion of thymic T cells reactive to self-peptides bound to MHC-encoded molecules of thymic APCs (Kappler et al. 1987; Kisielow et al. 1988). This

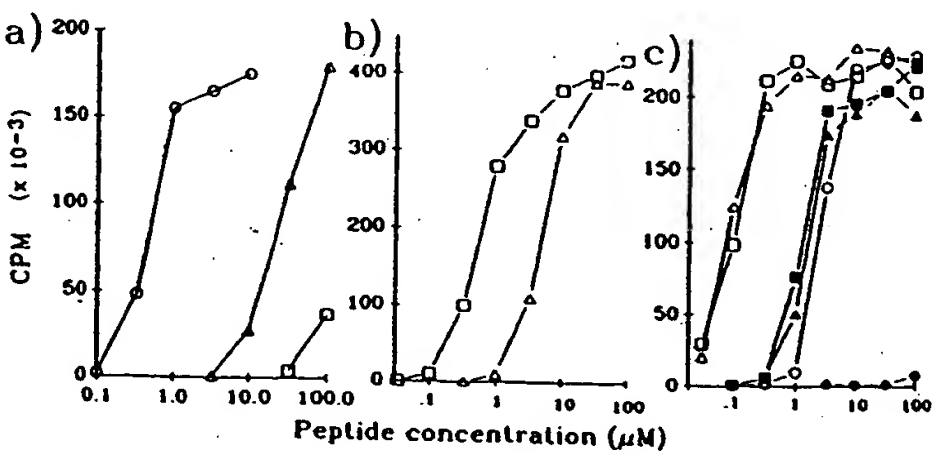

Figure 4. Hybridoma response to in vitro stimulation with peptides. Open symbols are live APCs, and filled symbols are fixed APCs. (*a*) 3D054.8 T-cell hybridoma and I-A^d-transfected L cells; (O) OVA, (Δ) joint peptide, (□) OVA-D. (*b*) 9C127 T-cell hybridoma and I-A^d-transfected L cells; (Δ) joint peptide, (□) p12-26. (*c*) 1E1 T-cell hybridoma and A20 cells; (O, ●) p1-102, (Δ, ▲) joint peptide, (□, ■) p12-26.

Table 4. Epitopes of BALB/c Hybridomas Immunized with *cI* p1–102

Immunization protocol	Number of hybrids			
	1–102 +	12–26 +	12–26⁻ tested with peptides	46–62 +
Alum i.p.	436	389	27	12
CFA i.p.	252	216	19	9
CFA s.c.	134	121	7	2
IFA i.p.	413	379	27	12
IFA s.c.	199	179	17	9
Total	1434	1284	97	44

mechanism can only delete those T cells that react to self-proteins that are expressed or travel to the fetal thymus. Expression in the fetal thymus of every self-T-cell epitope found in every protein in the organism would be extremely difficult. Even if tolerance is needed only for those proteins that are accessible to the immune system, and assuming efficient transport of all proteins to the fetal thymus, many proteins are only expressed after birth or later. T cells reactive to proteins expressed late in development would be able to leave the thymus and initiate autoimmune disease later in life. The fact that this does not occur argues for some sort of peripheral, continuing tolerance mechanism. We have found evidence for such a mechanism in that, by exposing a mouse to a T-cell epitope i.v. in saline or i.p. in IFA, the mouse later has a drastically diminished T-cell response to the same epitope when administered later using CFA as the adjuvant. This reduction or tolerance is epitope-specific, lasts for at least 6 weeks, and is not due to the induction of suppressor T cells. It also completely inhibits the antibody response to the epitope, presumably by blocking T-cell help.

We propose that the immune system in its resting state, i.e., unstimulated by an infectious agent or gross tissue damage, is normally in a tolerogenic state. All T cells that see antigen in that state are turned off either by clonal deletion or by clonal anergy. Only when the immune system is turned on by frank infection, by such

Table 5. IL-4-producing BALB/c Hybridomas from Mice Immunized with *cI* p1–102

Immunization protocol	Number of IL-4-producing clones/total clones	
	p12–26	p46–62
Alum i.p.	0/11	4/7
CFA i.p.	0/20	2/4
CFA s.c.	1/16	not recovered
IFA i.p.	2/30	4/11
IFA s.c.	1/15	5/5
Total	4/92 (4.3%)	15/27 (55.6%)

agents as the bacterial antigens and mitogens found in the mycobacteria, or by the presence of soluble cytokines, are T cells able to respond by proliferating, secreting IL-2, and helping B cells. Furthermore, all of the T-cell epitopes we have tested that are capable of eliciting an immune response are just those epitopes that can also serve to induce tolerance. Thus, it would appear that epitopes processed and presented in the absence of a second signal lead to tolerance. The observations presented here readily explain the classic low zone tolerance to soluble proteins.

Results from the present study clearly demonstrate a regulatory effect of competing epitopes present within the same peptide upon the immune response. Thus, a majority of the T-cell response to the joint peptide is

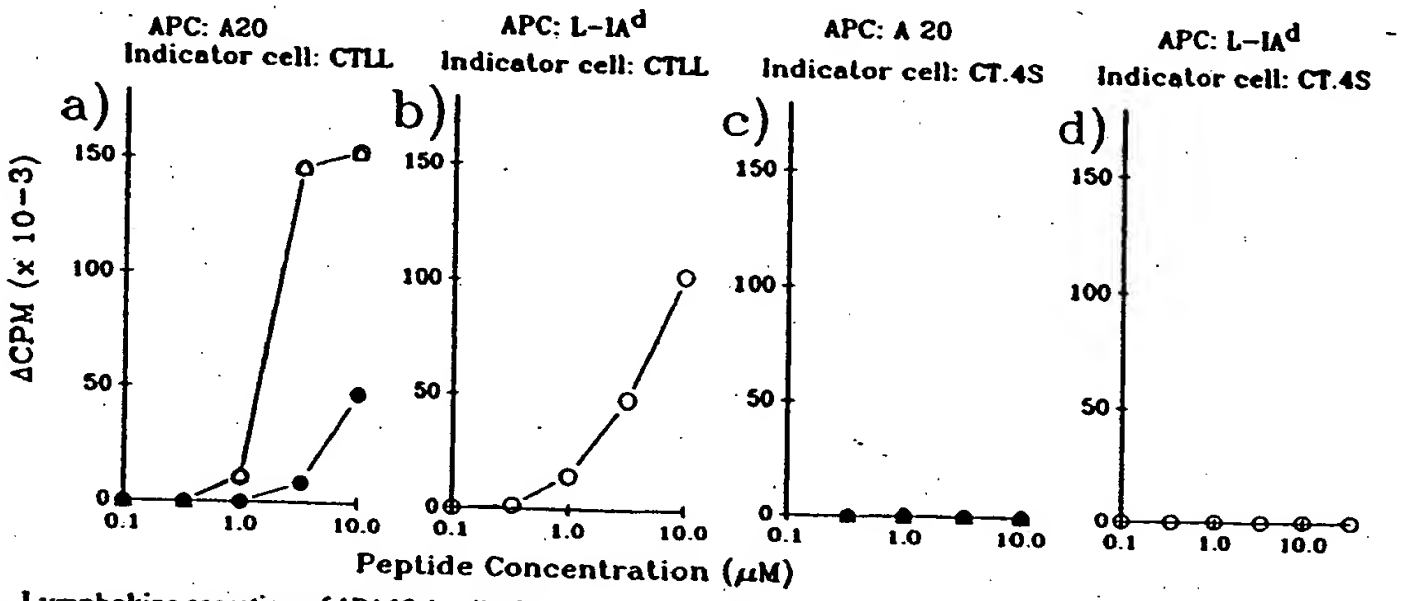

Figure 5. Lymphokine secretion of 1PA12-1 cells. Hybridoma response to in vitro stimulation with p12-26. Added to the indicator cells was (○) nothing, (●) anti-IL-2, (Δ) anti-IL-4. (*a*) IL-2 response with A20 cells; (*b*) IL-2 response with transfected L cells; (*c*) IL-4 response with A20 cells; (*d*) IL-4 response with transfected L cells.

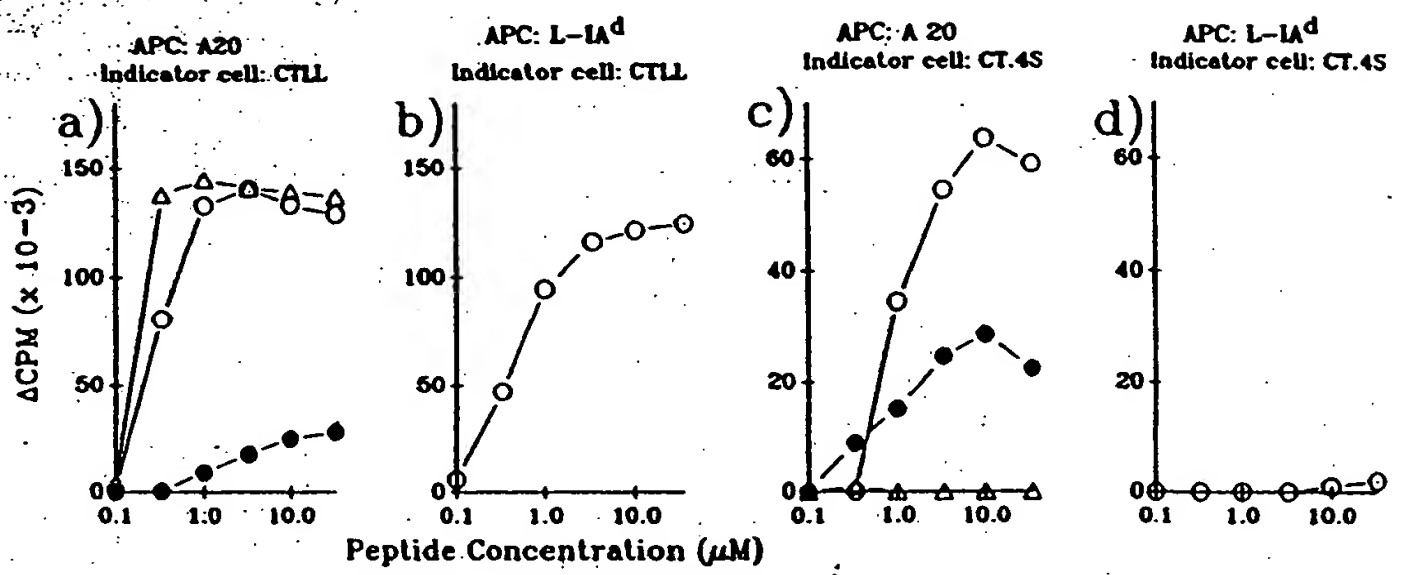

Figure 6. Lymphokine secretion of 1SI46-1.1 cells. Hybridoma response to in vitro stimulation with p46-62. Added to the indicator cells was (○) nothing, (●) anti-IL-2, (△) anti-IL-4. (*a*) IL-2 response with A20 cells; (*b*) IL-2 response with transfected L cells; (*c*) IL-4 response with A20 cells; (*d*) IL-4 response with transfected L cells.

directed toward the more immunogenic epitope (12-26), leading to an apparent suppression of the response to the less immunogenic epitope (OVA-D). It is also clear that competition occurs between T-cell epitopes during the induction of tolerance. Again, the more immunogenic epitope (12-26) is tolerized, and the less immunogenic epitope (OVA-D) is ignored. The T-cell response to the less immunogenic epitope can be elicited readily in mice tolerized with the joint peptide. Therefore, the processing mechanisms of APCs inducing immunity and those inducing tolerance seem to be the same, and the less immunogenic epitopes, such as OVA-D within the joint peptide 12-26-GPG-OVA-D, are hidden for both immunization and tolerization.

Many different cell types such as B cells, macrophages, and dendritic cells can serve in vitro as APCs (Chesnut and Grey 1981; Ziegler and Unanue 1981; Sunshine et al. 1983). The involvement of the different APCs used in an immune response in vivo are unknown. However, activation of T cells by the different APCs may vary because of differences in their endocytic and processing properties (Guidos et al. 1984). Thus, the immune response may be modulated by the involvement of different types of APCs during the activation of T cells. In addition, the type of adjuvant and the route of immunization may have selective effects on the APCs used in the immune response. In our study, we observed no significant difference in the T-cell response induced in BALB/c mice immunized with λ repressor p1-102 using different combinations of adjuvants and routes of immunization. However, over 50% of T-cell hybridomas specific for p46-62 secreted IL-4, whereas less than 5% of hybridomas specific for p12-26 did. The fact that all IL-4-producing hybrids also secrete IL-2 may be attributable to the fusion partner, BW5147, which has been shown to be capable of IL-2 production (Hagiwara et al. 1988) or, more likely, to the fact that the hybrids were initially screened for their abilities to secrete IL-2. We propose the existence of at least two different types of APCs that have different antigen-processing mechanisms to explain these results. One type of APC processes p1-102 in such a way that both epitopes 12-26 and 46-62 are presented, with epitope 12-26 being dominant. T cells activated by these APCs produce only IL-2, phenotypically similar to T-helper type 1 (T_H1) cells. The other type of APC processes p1-102 in such a way that the 46-62 epitope predominates. T cells activated by this type of APC often secrete IL-4, phenotypically similar to T_H2 cells. An alternate explanation is that a single type of APC may be differentially activated to alter both its processing activity, i.e., to produce and present different epitopes, and its concomitant different second signals that alter the activation of responding T cells in such a way that their lymphokine production and thus their effect on the qualitative nature of the antibody response is altered. These possibilities are currently being explored.

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Immunology

Suppression of type II collagen-induced arthritis by intragastric administration of soluble type II collagen

(orally induced immunologic unresponsiveness/autoimmunity)

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ABSTRACT Although oral administration of protein antigens may lead to specific immunologic unresponsiveness, this method of immunoregulation has not been applied to models of autoimmune disease. Type II collagen-induced arthritis is an animal model of polyarthritis induced in susceptible mice and rats by immunization with type II collagen, a major component of cartilage. Intragastric administration of soluble type II collagen, prior to immunization with type II collagen in adjuvant, suppresses the incidence of collagen-induced arthritis. Administration of denatured type II collagen has no observable effect on the incidence or severity of the disease. The overall magnitude of the antibody response is not significantly reduced in collagen-fed mice as compared to controls. While the isotype distribution of the anti-collagen antibodies is similar in the two groups, there is a tendency toward reduced IgG2 responses in the collagen-fed mice.

Type II collagen-induced arthritis (CIA) is an animal model of polyarthritis induced in susceptible mice and rats by immunization with type II collagen (1, 2). Type II collagen is the major matrix protein of hyaline cartilage. The similarity of the histopathologic changes observed in CIA to those seen in human rheumatoid arthritis has centered interest on the contribution of collagen autoimmunity to the pathogenesis of the human disease. Although humoral and cellular immunity to type II collagen have been shown in CIA, the precise contribution of each to the development of disease has not been established. While T cells have been shown to recognize undenatured and denatured type II collagen (3), the humoral response is restricted to the undenatured, nonrepeating helical antigenic determinants of the collagen molecule (3, 4). Development of disease after immunization with type II collagen in mice is restricted by the major histocompatability type (5). Although many mouse strains produce a vigorous humoral immune response to type II collagen, only mice of the H-2^q haplotype develop arthritis. The induction of acute manifestations of CIA by the transfer of anti-type II collagen antibodies (hereafter referred to as anti-collagen antibodies) from arthritic to normal mice emphasizes the critical role of anti-collagen antibodies in the pathogenesis of CIA (6).

Several attempts to modulate the disease have led to antigen-specific suppression of collagen immunity and decreased incidence of arthritis. Induction of arthritis is suppressed by prior i.v. injection of type II collagen-coupled spleen cells (7). In rats, spleen cells from donors receiving type II collagen-coupled rat erythrocytes transfer antigen-specific suppression of CIA (8). Intravenous administration of soluble type II collagen suppresses induction of arthritis in rats and mice when given before primary immunization (9–11) or during the afferent phase of disease induction, 7–10 days after primary immunization (12).

Oral presentation of antigen is the earliest recorded method for experimentally inducing specific antigenic unresponsiveness (13, 14). This route of antigen administration can lead to immunity or tolerance, depending on the dose, number of feedings, and the form of antigen used (15). T-dependent, but not T-independent, antigens can lead to the induction of oral tolerance (16), and the immune response to collagen is T dependent (17, 18). The studies reported here show that intragastric administration of soluble type II collagen suppresses the induction of CIA in mice. It is, therefore, possible to suppress an experimental autoimmune disease by orally induced unresponsiveness.

MATERIALS AND METHODS

Antigens and Immunizations. DBA/1 Lac J male mice were purchased from The Jackson Laboratories and immunized at age 8–14 weeks. Type II collagen was solubilized from fetal bovine articular cartilage by limited proteolysis with pepsin, essentially according to the technique of Trentham *et al.* (1). Collagen purity was assessed by analysis of amino acids by Genetic Design (Watertown, MA) and by $NaDodSO_4$/PAGE (19). Type II collagen was dissolved in 0.01 M acetic acid at 4°C prior to use. Denatured type II collagen was prepared by incubation at 56°C for 45 min. Intragastric feedings (0.5 ml) were administered with a ball-tipped feeding needle. Control animals were fed 0.01 M acetic acid (0.5 ml). Mice were immunized parenterally by intradermal injection of 300 μg of type II collagen emulsified in Freund's adjuvant containing heat-killed mycobacteria at 4 mg/ml (strains C, DT, and PN; Ministry of Agriculture, Fisheries and Food, Weybridge Surrey, England). Mice were boosted with 100 μg of type II collagen i.p. on day 21.

Assessment of Arthritis. Mice were observed two or three times each week for presence of distal joint swelling and erythema. Swelling was quantitated by measuring thickness of foot and width of ankle with a constant tension caliper (Dyer, Lancaster, PA). A mouse was considered arthritic when swelling and erythema were observed on consecutive measurement dates in at least one paw. In addition, clinical severity of arthritis was assessed by creation of an arthritic index. Each limb was subjectively graded on a scale of 0–3 (0, absence of arthritis; 1, mild swelling and erythema; 2, swelling and erythema of both tarsus and ankle; 3, ankylosis and bony deformity). A maximum arthritic index (MAI) was obtained for each mouse by summing the greatest score recorded for each limb (0, no disease; 12, highest possible

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Abbreviations: CIA, collagen-induced arthritis: MAI, maximum arthritic index.

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score) (9, 20). The MAI for each group was calculated according to the formula:

$$\frac{\text{mean MAI} \times \text{number of arthritic mice}}{\text{number of mice in the group}}$$

Measurement of Anti-Collagen. Antibodies to type II collagen in immune sera were measured by ELISA (21). To obtain μg/ml values of anti-collagen antibodies of each isotype, a mouse immunoglobulin reference serum (Miles Scientific, Naperville, IL) containing known amounts of each isotype was used as a standard (22). A mouse anti-collagen standard immunoglobulin preparation was purified from the sera of arthritic mice on a type II collagen-Sepharose column (23). Immulon 2 plates (Dynatech, Alexandria, VA) coated with rabbit anti-mouse immunoglobulin at 100 μg/ml in 0.016 M boric acid/0.15 M NaCl, pH 8, were blocked with the same buffer containing 2% (vol/vol) horse serum. The plates were washed and incubated with serial dilutions of affinity purified mouse anti-collagen and reference serum. The assay was developed by the addition of peroxidase-conjugated rabbit anti-mouse IgG1, IgG2a, IgG2b, IgG3, IgA, or IgM (Miles Scientific), and the substrate ABTS [2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid), Zymed Laboratories, South San Francisco, CA]. The cross reactivity of these sera was tested with purified mouse myeloma subclass proteins (Litton Bionetics, Organon Teknica, Charleston, SC) and found to be <10%. Absorbances at 405 nm were determined with a vertical beam spectrophotometer (Artek Systems, Farmingdale, NY). The absorbance values obtained were used to construct a standard curve for each isotype using a computer program that does a least squares fit correlating absorbance with concentration. Curves were generated through a third-order equation in which absorbance is the independent variable and, after subtracting background, the intercept is assumed to be zero. The μg/ml values obtained for anti-collagen antibodies of each isotype in the affinity-purified mouse anti-collagen standard were then used to create standard curves with type II collagen-coated plates. Anti-collagen antibodies of each isotype in immune sera were quantitated by titration on type II collagen-coated plates developed with the rabbit anti-mouse isotyping reagents described above.

Anti-collagen antibodies in immune sera were also measured by solid-phase RIA (24). Polyvinyl chloride microtiter plates (Dynatech, Alexandria, VA) were coated with type II collagen at 100 μg/ml, blocked with 2% (vol/vol) horse serum, and incubated with dilutions of immune sera. The plates were developed with ^{125}I-labeled rabbit anti-mouse Fab. Individual wells were cut out and counted in a γ counter. Affinity-purified mouse anti-collagen antibodies were used as a standard.

Statistical Analysis. Comparisons of means were performed by Student's t test. Arthritis incidences were compared with the Fisher exact test. P values given are for two tailed tests.

RESULTS

Resistance to Arthritis Induction After Feeding of Soluble Type II Collagen. Soluble type II collagen was administered intragastrically to CIA-susceptible DBA/1 Lac J mice prior to intradermal immunization with type II collagen in complete Freund's adjuvant. Twelve feedings (500 μg each) of type II collagen in 0.5 ml of 0.01 M acetic acid during 6 weeks significantly reduced the incidence of arthritis (Fig. 1*A*; $P < 0.004$ for each time point beyond day 30). For the group, the MAI on day 58 was 1.9 as compared to 3.9 in controls (Fig. 1*B*). For those mice in the type II collagen-fed group that did become arthritic the mean day of onset (day 44 ± 4) was not significantly delayed compared to controls (day 41 ± 2). The clinical severity of the arthritis in these animals also did not differ significantly from that in the arthritic controls (mean MAI ± SEM on day 58 of type II collagen fed animals, 5.6 ± 0.76; meap MAI controls ± SEM, 4.8 ± 0.36; P, 0.34).

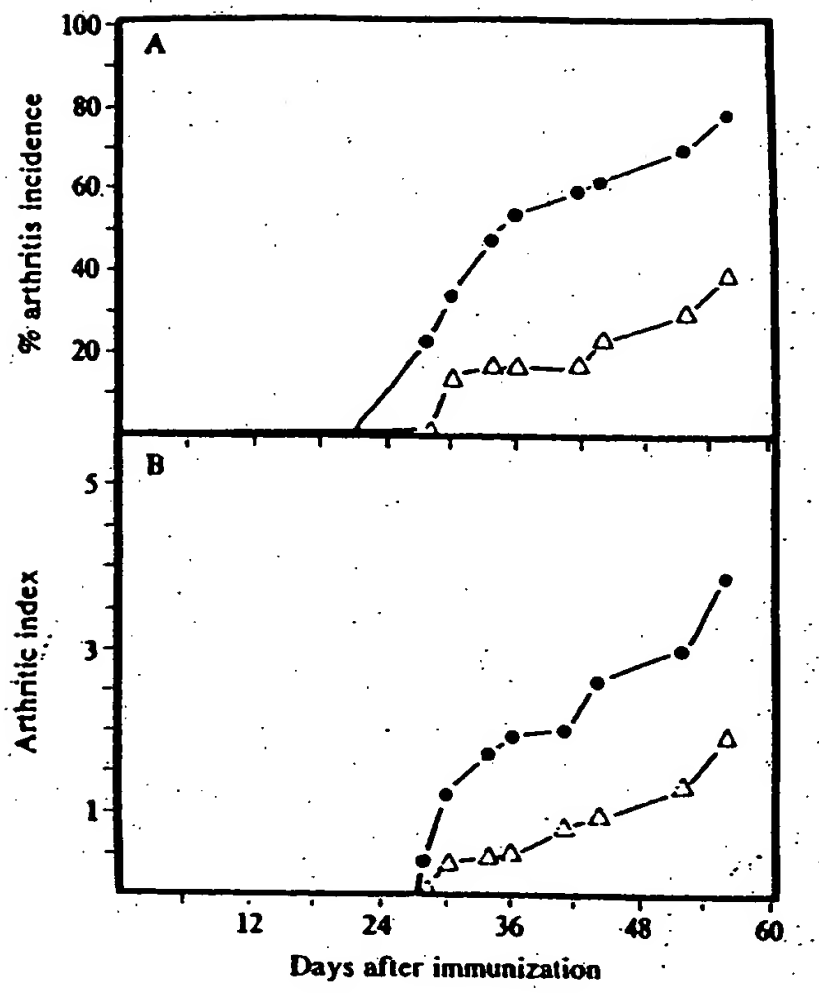

FIG. 1. (*A*) Incidence of arthritis after intragastric administration of 500 μg of type II collagen in 0.5 ml of 0.01 M acetic acid 12 times during 6 weeks (summary of six experiments). △, type II collagen (n = 32); ●, controls (n = 51). (*B*) Clinical severity of arthritis. △, type II collagen (n = 32); ●, controls (n = 51).

When both the interval and the number of feedings were decreased, it was found that eight intragastric administrations of type II collagen given over a 2-week period were sufficient to reduce the incidence of CIA significantly (Fig. 2*A*). Administration of denatured type II collagen, however, had no effect on the incidence (Fig. 2*B*) or the severity of the arthritis (MAI for the group fed denatured type II collagen, 3.4; MAI controls, 3.3). When each intragastric administration consisted of 3 mg, rather than 0.5 mg, of type II collagen no suppression of CIA was observed (Fig. 2*C*). The clinical severity of the arthritis observed in these animals was slightly higher (MAI, 4.4) than that observed in the corresponding controls (MAI, 3.7).

Serum Antibody Levels in Type II Collagen-Fed and Control Mice. Table 1 shows the distribution of immunoglobulin isotypes in the anti-collagen response in sera taken during the onset of disease on day 35 after primary immunization. The results are presented separately for animals with and without arthritis. The total antibody levels given are the sums of those for the individual isotypes. None of the isotypes showed statistically significant differences between arthritic and nonarthritic mice in the control group but IgG2a and IgG2b tended to be higher in the arthritic mice. IgG2b levels in type II collagen-fed mice were significantly lower than those in arthritic control mice, while IgG2a levels were also reduced, but not significantly different from those in control mice. The IgG1, IgG3, and IgA responses were lower but did not differ significantly in fed and control mice.

Since IgG2a and IgG2b were most prominent in the anti-collagen response, it was of interest to determine the

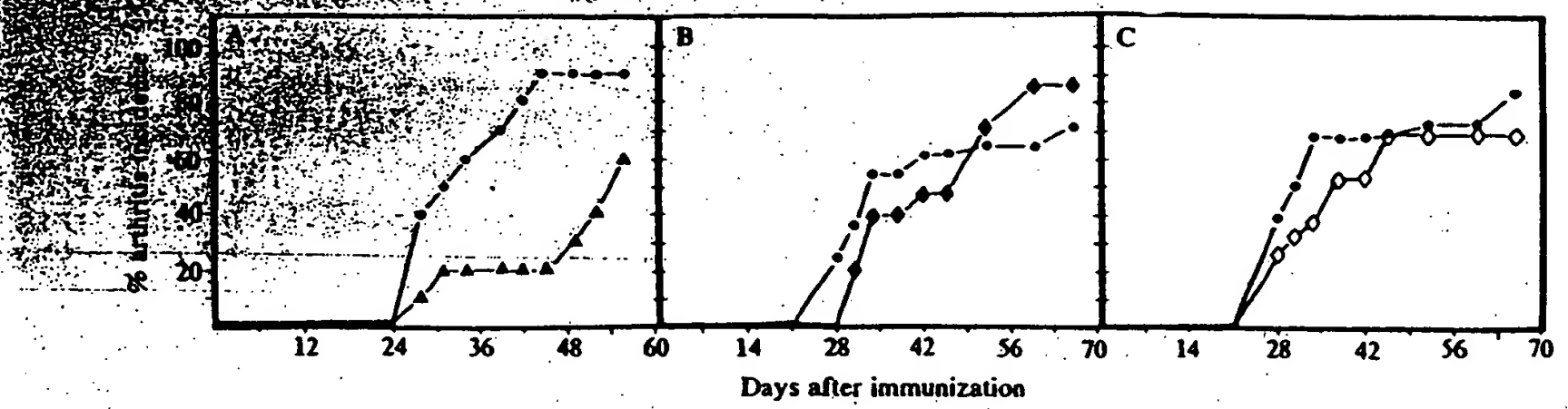

FIG. 2. Incidence of arthritis after intragastric administration of type II collagen. (*A*) type II collagen (500 μg) in 0.5 ml of 0.01 M acetic acid eight times during 2 weeks (▲, $n = 10$). Controls 0.5 ml of 0.01 M acetic acid (●; $n = 10$). (*B*) Denatured type II collagen (500 μg) in 0.5 ml of 0.01 M acetic acid 12 times during 6 weeks (◆, $n = 15$). Controls (as in *A*) (●, $n = 28$). (*C*) type II collagen (3 mg) in 1 ml of 0.01 M acetic acid 12 times during 6 weeks (◇, $n = 19$). Controls had 1 ml of acetic acid (●, $n = 18$). Mice were immunized with type II collagen in complete Freund's adjuvant 3 days after the last dose of type II collagen.

level of these antibodies during the afferent phase of disease induction prior to onset of arthritis, as well as later in the course of the disease (Table 2). The IgG2a and IgG2b levels measured on day 20 after immunization were similar in all of the mice. In spite of the resistance to disease induction in type II collagen-fed mice, their IgG2a antibody levels were not significantly lower than those of control mice. However, the tendency to reduced IgG2a and IgG2b antibody levels observed during the onset of the disease (day 35) persisted, even in sera taken on day 60 after immunization. Measurement of total serum anti-collagen antibodies by RIA gave similar results. In a group of control ($n = 4$) and type II collagen-fed ($n = 7$) mice, anti-collagen levels on day 20 were 78 ± 21 and 99 ± 35 μg/ml (mean ± SEM), respectively. Sera from arthritic control mice ($n = 9$) taken on day 60 measured 244 ± 46 μg/ml, while nonarthritic type II collagen-fed mice measured 156 ± 19 μg/ml (mean ± SEM; $n = 10$; $P = 0.17$).

DISCUSSION

It has been postulated that immunologic unresponsiveness is induced after intragastric administration of antigen by the separation in the gut of tolerogenic, monomeric forms of antigen from immunogenic large molecular weight aggregates (25). After intragastric administration of soluble bovine serum albumin (25) or ovalbumin (26), only monomeric antigen could be detected in the sera of fed animals. Very small quantities of intact native proteins or small fragments bearing antigenic determinants of the native protein appear to be absorbed. In the present experiments, intragastric administration of undenatured, but not denatured, type II collagen leads to suppression of CIA. This is consistent with evidence that the immune response to type II collagen in mice is directed against the undenatured helical antigenic determinants (4, 27) and that only immunization with undenatured type II collagen readily induces arthritis (23, 27).

Eight feedings of soluble type II collagen over a 2-week period are as effective in suppressing CIA as 12 feedings over 6 weeks. In contrast, administration of large doses of type II collagen in each feeding does not result in suppression of CIA. Results from other investigators (reviewed in ref. 15) also suggest that the induction of unresponsiveness by the oral route is strikingly dose dependent. Continued feeding reduces the absorption of antigen, probably as a result of local immunity (15). It is possible that the high dose of type II collagen used here may have immunized the recipient, although no anti-collagen antibodies, even of the IgA isotype, have been detected in sera taken after feeding. It is of interest that the severity of the disease observed in the fed animals that do become arthritic does not differ from that in controls.

It has been reported that feeding of antigen subsequent to parenteral immunization either has no suppressive effect or boosts antibody production (16). Others suggest, however, that continued feeding of small doses of antigen may lead to systemic unresponsiveness in spite of initial priming (15). Repeated oral administration of ovalbumin can prevent a secondary antibody response in primed mice (28). In the present studies (data not shown) eight intragastric administrations of type II collagen given between days 10 and 29 after immunization with type II collagen in complete Freund's adjuvant did not result in decreased incidence or severity of CIA.

It has been suggested that CIA is an autoimmune disease initiated by the binding of antibody to autologous type II collagen in the joint (29). Complement is required for the development of CIA (30). Onset of disease in susceptible mouse strains is associated with a predominance of IgG2a anti-collagen whereas resistant strains mount a relatively deficient IgG2a response (22). This is in agreement with the fact that IgG2 is the most efficient member of the mouse IgG class in the fixation of complement by the classical pathway (31). The IgG2 anti-collagen response is, therefore, of par-

Table 1. Effect of type II collagen feeding on isotype distribution of serum anti-collagen on day 35 after immunization

Pretreatment	Arthritis	No. of mice	Anti-collagen isotype, μg/ml					Total immunoglobulin, μg/ml
			IgG1	IgG2a	IgG2b	IgG3	IgA	
Acetic acid-fed control	+	6	52 ± 14	437 ± 108	107 ± 17	10 ± 3	48 ± 14	653 ± 132
	−	4	49 ± 21	169 ± 31	42 ± 11	6 ± 3	25 ± 7	291 ± 53
	Total	10	51 ± 11*	330 ± 77†	81 ± 15‡	8 ± 2§	39 ± 9¶	508 ± 98‖
Collagen fed	+	2	47 ± 7	122 ± 32	30 ± 9	4 ± 3	16 ± 3	227 ± 18
	−	6	34 ± 16	136 ± 8	37 ± 23	5 ± 4	16 ± 1	219 ± 42
	Total	8	44 ± 7*	126 ± 24†	32 ± 9‡	4 ± 2§	16 ± 2¶	221 ± 31‖

Results are mean ± SEM. +, Mice with arthritis. −, Mice without arthritis.
*P, 0.64; †P, 0.09; ‡, 0.03; §P, 0.19; ¶P, 0.12; ‖P, 0.06.

Table 2. IgG2a and IgG2b serum anti-collagen prior to and late in the development of CIA in type II collagen-fed and control mice

Day	Pretreatment	Arthritis	No. of mice	Anti-collagen isotype, μg/ml IgG2a	IgG2b
20	Acetic acid-fed control	−	5	70 ± 8*	19 ± 6†
	Collagen fed	−	8	98 ± 36*	16 ± 4†
60	Acetic acid-fed control	+	9	448 ± 134‡	32 ± 7§
	Collagen fed	+	4	387 ± 143	59 ± 17
		−	9	294 ± 166‡	12 ± 2§

Results are mean ± SEM. +, Mice with arthritis. −, Mice without arthritis.
*P, 0.6; †P, 0.7; ‡P, 0.49; §P, 0.06.

ticular importance for the development of CIA. The possibility exists that resistance to induction of CIA observed after intragastric administration of type II collagen results from a decrease in the magnitude of the IgG2 response or a switch in isotype predominance. We have found that while the overall anti-collagen response is slightly, but not significantly lower, IgG2a remains the predominant isotype in both fed and control mice. In addition, although much lower in magnitude than the IgG2a response, the IgG2b anti-collagen response is significantly lower in nonarthritic type II collagen-fed mice than in arthritic control mice.

There are at least two possible mechanisms for the resistance to arthritis induction observed after feeding of soluble type II collagen. Mattingly and Waksman (32) found that feeding sheep erythrocytes for 2 weeks resulted in systemic unresponsiveness. Within 2 days after feeding, suppressor T cells appeared in the Peyer's patches and mesenteric lymph node, but were undetectable in these locations and present in the spleen and thymus after 4 days. Suppressor T cells have also been shown, by adoptive transfer, in the mesenteric lymph node and spleen of animals suppressed by oral administration of soluble proteins or haptens in several other experimental systems (33–38). In the present system collagen-specific suppressor T cells could have suppressed antibody production, particularly of the IgG2 class, or might have prevented the sensitization of T cells directly involved in the initiation and maintenance of arthritis. Alternatively, production of anti-idiotypic autoantibody has been postulated to depress specificially the IgM and IgG immune response after systemic challenge of fed animals with an immunizing dose of antigen (39, 40). Although in the present study the effect of type II collagen feeding on the magnitude of the humoral anti-collagen response is not marked, it is still possible that anti-idiotypic regulation could have suppressed a part of the response critical for the induction of CIA by virtue of its specificity.

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SUPPRESSION OF EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS BY ORAL ADMINISTRATION OF MYELIN BASIC PROTEIN AND ITS FRAGMENTS[1]

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We report that experimental autoimmune encephalomyelitis, a T cell-mediated autoimmune disease studied as a model for multiple sclerosis, can be suppressed in Lewis rats by the oral administration of myelin basic protein (MBP). Both the clinical and histopathologic manifestations of the disease were suppressed in a dose-dependent manner. In addition, proliferative responses to MBP and, to a lesser extent, serum levels of anti-MBP antibody were suppressed by feeding MBP. Suppression of clinical and histologic disease was observed whether animals were fed MBP before or after disease induction, although suppression was more complete when rats were fed before immunization. Disease was also suppressed by the oral administration of either encephalitogenic or nonencephalitogenic fragments and decapeptides of the MBP molecule, with more complete suppression observed when nonencephalitogenic fragments were fed, suggesting that suppressor determinants exist in the MBP molecule distinct from the encephalitogenic region. The oral administration of a non-disease-inducing portion of an autoantigen represents an antigen-specific method by which an experimental autoimmune disease can be immunoregulated.

Experimental autoimmune encephalomyelitis (EAE)[3] is a T cell-mediated autoimmune disease directed against myelin basic protein (MBP) and has been studied in several mammalian species as a model for the human demyelinating disease multiple sclerosis (1). Several studies have focused on methods to suppress the development and severity of EAE. For example, it has been demonstrated that suppression of the disease is possible by non-antigen-specific methods such as treatment with cyclophosphamide (2) or injection of monoclonal antibodies directed against T cell subsets (3, 4). In addition, various antigen-specific methods have been used to suppress EAE, including systemically administered antigen or antigen conjugated to lymphoid cells (5–7).

An effective and long recognized method of inducing immunologic tolerance is the oral administration of antigen (see Reference 8 for review), which was first demonstrated by Wells for hen's egg proteins in 1911 (9). Oral induction of immunologic unresponsiveness has been demonstrated for a number of T-dependent, but not T-independent, antigens (8, 10) and we have previously investigated oral tolerance to viruses (11). Orally induced tolerance has been shown in several instances to be the result of the generation of antigen-specific suppressor T cells (11–19), although other mechanisms including antiidiotypic antibodies and immune complexes have also been implicated as being responsible for the induction of oral tolerance (20–22). In the present report, we have studied the effect of feeding MBP on the susceptibility to and severity of acute monophasic EAE in the Lewis rat. In addition, because only a small segment of the MBP molecule is known to be encephalitogenic and the remainder is unable to induce disease (23), we also examined the effect of oral administration of peptide fragments of MBP on the development of EAE. Our results show that the oral route of antigen administration suppresses not only disease but also various immune responses to MBP and that suppression of EAE is also possible by feeding nonencephalitogenic fragments of the MBP molecule.

MATERIALS AND METHODS

Rats. Lewis rats were obtained from the Charles River Laboratory (Wilmington, MA). Animals were used at 6 to 8 wk of age.

Antigens. Guinea pig and bovine MBP were purified from brain tissue by the method of Diebler et al. (24). Fragments of guinea pig MBP were generated by limited pepsin digestion at 25°C, pH 3.0, and were separated by ion exchange chromatography as described (25). The peptides were run on phosphoric acid-urea sodium dodecyl sulfate-polyacrylamide gels (26) to verify their homogeneity. The bovine encephalitogenic decapeptide (Ala-Gln-Gly-His-Arg-Pro-Gln-Asp-Glu-Asn) and the decapeptide S79, which differs from the encephalitogenic decapeptide by a single amino acid substitution (Gly for Asn) at the carboxyl terminus, were synthesized using an Applied Biosystems 430A peptide synthesizer.

Induction of oral tolerance. Rats under light ether anesthesia were fed MBP or fragments in 0.25 ml of phosphate-buffered saline (PBS) solution by using a syringe fitted with a 20-gauge ball point needle. Control animals were fed equal amounts of bovine serum albumin (BSA) or saline alone.

Immunizations. EAE was induced in Lewis rats by immunization with guinea pig MBP emulsified in complete Freund's adjuvant (CFA). A total of 0.4 ml of emulsion containing 50 μg of MBP in 0.2 ml saline and 0.2 ml of CFA was injected into the hind footpads of each rat. Disease was characterized by hind limb paralysis and incontinence, usually between days 12 and 15 after immunization, and in all cases rats recovered by day 16. Clinical scoring was as follows: 1 = tail weakness; 2 = tail weakness plus hind limb weakness; 3 = hind limb paralysis plus incontinence; and 4 = Moribund.

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[3] Abbreviations used in this paper: EAE, experimental autoimmune encephalomyelitis; MBP, myelin basic protein; CFA, complete Freund's adjuvant; PBST, phosphate-buffered saline containing 0.05% Tween 80.

Histology. Rats were killed on day 16 after immunization and brains were removed and fixed in a solution of 3% formaldehyde, 60% ethanol, and 4% acetic acid. Slides of paraffin-embedded tissue were prepared from each individual rat and 10-μm sections were stained with hematoxylin and eosin. Numbers of perivascular inflammatory foci were quantified from coded slides by established procedure (27).

Proliferation assay. Ten days after immunization, some of the rats from each experimental group were killed, the popliteal lymph nodes were removed, and single cell suspensions were prepared. A volume of 0.2 ml containing 4×10^5 cells in RPMI 1640 containing 2% glutamine, 1% penicillin/streptomycin, 5×10^{-5} M 2-mercaptoethanol, and 5% fetal calf serum was added to each microwell and MBP was added at 50 μg/ml. The cells were cultured for 72 hr, each well was pulsed with 1 μCi of tritiated thymidine, and cells were cultured for another 24 hr. Cultures were harvested onto fiberglass filters using a multiharvester and counted using standard liquid scintillation techniques. All cultures were performed in triplicate and results were expressed as stimulation indexes (experimental cpm/control cpm).

Measurement of serum antibody. An enzyme-linked immunosorbent assay was used to measure the level of serum anti-MBP antibody in rats. A volume of 0.1 ml of MBP solution (0.05 mg/ml in PBS) was added to each microwell and was incubated for 3 hr at 37°C. Wells were washed with PBS containing 0.05% Tween 80 (PBST) and were blocked overnight at 4°C with 5% BSA in PBS, pH 9.0. After washing with PBST, rat sera (diluted in PBS) were added and incubated for 3 hr at room temperature and, after washing with PBST, secondary antibody (peroxidase-conjugated goat anti-rat IgG) was added for 1 hr at room temperature. Substrate was added and the reaction was stopped with 0.1 M NaF. Plates were read at 450 nm on a Titertek multiscan. Absorbance at 450 nm was also determined from serum from rat immunized with only CFA and was subtracted from all values as background.

RESULTS

Oral tolerance of EAE is dose-dependent. The first series of experiments investigated the effect of the number of feedings and the dose of MBP on the suppression of disease. Rats were fed various amounts of MBP either once 7 days before (day −7) the day of immunization (day 0) or three times on days −14, −7, and 0. The results (Table I) demonstrate that feeding MBP to rats suppresses EAE and that orally induced suppression is dose dependent. Multiple 500-μg feedings resulted in complete suppression of disease and were more effective than a single feeding at this dose. In addition to clinical manifestation of EAE, histologic evidence of disease in rats was examined. Rats were sacrificed 16 days after immunization and brains were removed and fixed in formalin solution. As shown in Table I, feeding rats 500 μg of MBP on days −14, −7, and 0 caused a marked decrease in the number of inflammatory lesions in the brain. A moderate decrease was found in animals fed 100 μg and no significant reduction of inflammation was found in rats fed 25 μg of MBP.

Effect of feeding MBP before or after immunization. A second series of experiments investigated the effect of feeding MBP before or after immunization with MBP to determine whether orally induced suppression is affected by previous exposure to antigen. For these experiments, animals were fed 500 μg of MBP three times within a narrower time frame either before (days −7, −5, and −2) or after (days +2, +5, and +7) active induction of disease (immunization with MBP). The results (Table II) show that the clinical expression of disease is suppressed whether animals were fed MBP before or after sensitization, the effect being more complete when antigen was fed before immunization. However, histologic examination revealed a marked reduction of perivascular infiltrates in rats fed MBP either before or after sensitization to MBP. Greater than 60% suppression of clinical disease was also observed when rats were fed 500 μg of MBP three times beginning on day +5 or on day +7 after immunization (data not shown).

Further experiments were conducted to determine the persistence of orally induced protection against EAE. After receiving 500 μg of MBP on days −7, −5, and −2, rats were immunized at various lengths of time after the last feeding. EAE was completely suppressed in rats at 4 wk after feeding; by 8 wk 50% of animals were again susceptible to disease (data not shown).

To determine whether suppression of EAE by feeding MBP was specific for MBP and not due to a nonspecific effect of a basic protein, rats were fed 500 μg of histone three times (days −7, −5, and −2) and immunized with MBP. Feeding histone had no effect on the incidence of EAE (data not shown).

Effect of feeding MBP on immune responses to MBP. The effect of oral administration of MBP on cellular and humoral immune responses to MBP was also examined. Proliferative responses to MBP were studied after feeding rats different doses of MBP and following feeding at different times with respect to immunization. Rats were killed 10 days after immunization and proliferative responses of draining lymph node cells were determined. The results (Fig. 1) demonstrate that feeding MBP before immunization caused a pronounced decrease (75 to 92% suppression) of the proliferative response to MBP. Suppression of proliferation, unlike suppression of disease, occurred at all doses and feeding regimes tested. Feeding MBP after immunization was also effective in suppressing the proliferative response to MBP (Fig. 2). Orally induced suppression of the proliferative re-

TABLE I

Effect of feeding dose on orally induced suppression of EAE

MPB Feeding (μg)[a]	Clinical Disease[b]	Histologic Score[c]
Control	19/22	9.2 ± 5.8
Day −7		
25	3/5	ND
100	2/5*	ND
500	3/10***	ND
Days −14, −7, 0		
25	3/5	7.4 ± 5.2
100	2/5*	3.2 ± 1.9
500	0/10***	0.2 ± 0.4

[a] Rats were fed various doses of MBP on the indicated days and immunized with 50 μg of MBP in CFA (200 μg of *M. tuberculosis*) on day 0. Shown are the number of diseased rats of the total number tested. Immunized controls were fed BSA or saline. Clinical disease in controls and in fed animals consisted of hind limb paralysis and incontinence.

[b] Groups were compared with immunized controls by χ^2 analysis with one degree of freedom. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

[c] Rats were killed on day 16 after immunization and brains were removed and fixed. Shown are the average number of perivascular inflammatory foci per group ± SD. ND, not determined.

TABLE II

Effect of feeding MBP to rats before or after immunization on the development of EAE

MBP Feeding[a]	Clinical Disease[b]	Histologic Score[c]
Control	23/26	21.6 ± 5.1
Days		
−7, −5, −2, +2, +5, +7	0/5***	0.2 ± 0.4
−7, −5, −2	0/17***	0
+2, +5, +7	4/10**	1.4 ± 2.3

[a] Rats were fed 500 μg of MBP on the indicated days and immunized with 50 μg of MBP in CFA on day 0. Immunized controls were fed BSA or saline. Clinical disease in controls and fed animals consisted of hind limb paralysis and incontinence.

[b] Groups were compared with immunized controls by χ^2 analysis with one degree of freedom. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

[c] See Table I.

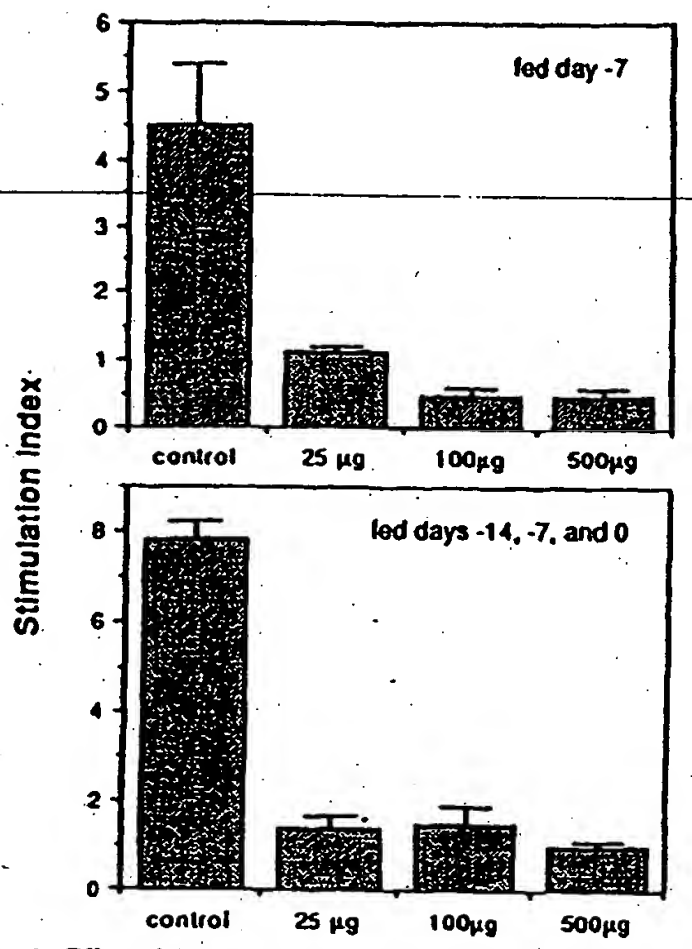

Figure 1. Effect of dose and number of feedings of MBP on proliferative response to MBP. Animals were fed various doses on the indicated days and then immunized with 50 µg of MBP in CFA on day 0. Proliferative response of popliteal lymph nodes was determined 10 days later.

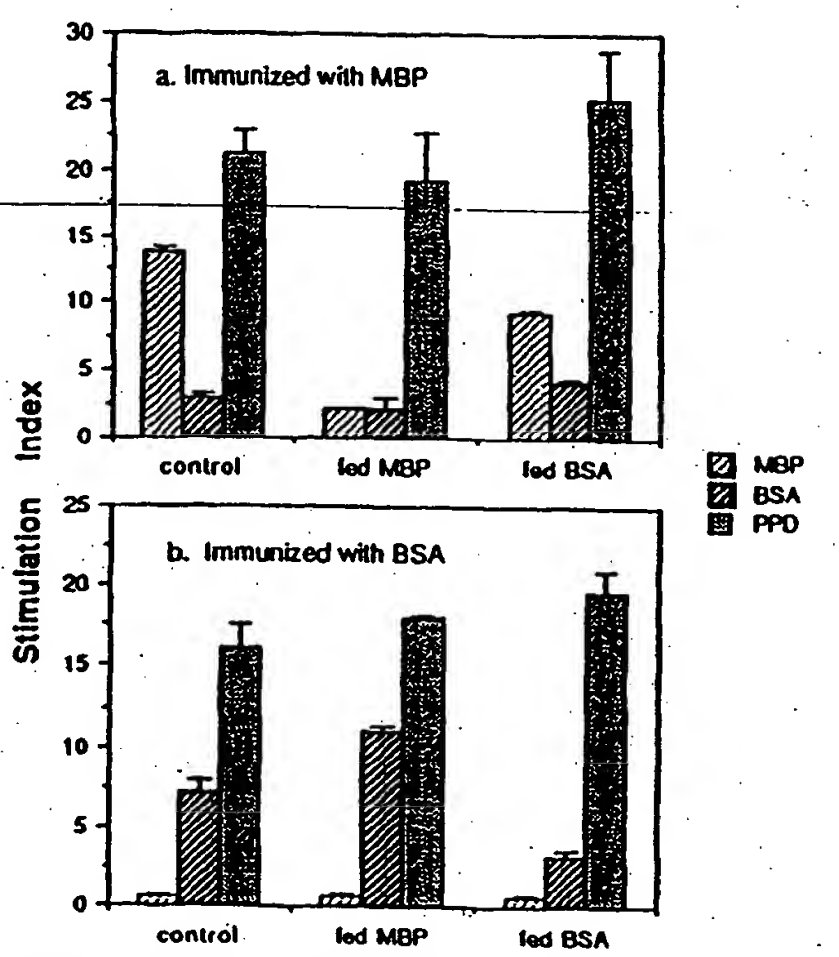

Figure 3. Antigen specificity of orally induced suppression of the proliferative response. Animals were fed 500 µg of MBP or BSA on days −7, −5, and −2 and immunized with 100 µg of MBP or BSA in CFA on day 0. Nine days after immunization, lymph nodes were removed and proliferative response to MBP, BSA, and a purified protein derivative of *Mycobacterium tuberculosis* (PPD) (all at 50 µg/ml) was determined.

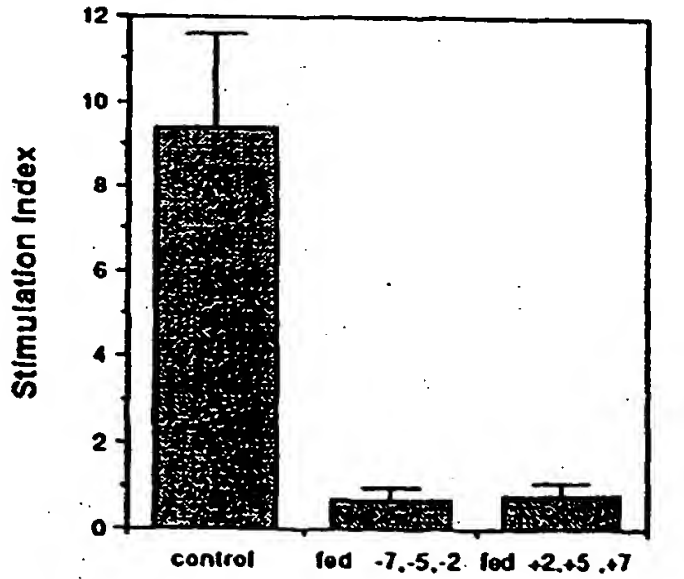

Figure 2. Effect of feeding MBP before or after immunization on proliferative response to MBP. Animals were fed 500 µg of MBP on the indicated days and immunized with 50 µg of MBP in CFA on day 0. Proliferative response of popliteal lymph nodes was determined on day 10 after immunization.

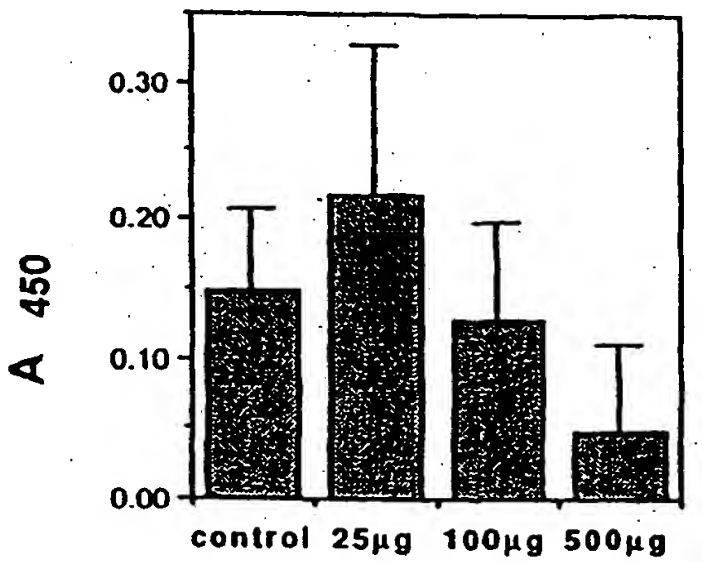

Figure 4. Effect of feeding MBP on the production of serum anti-MBP antibody. Animals were fed the indicated amounts of MBP on days −14, −7, and 0 and immunized with 50 µg of MBP in CFA on day 0. Rats were bled 16 days after immunization and serum antibody levels were determined by enzyme-linked immunosorbent assay. Shown are the relative absorbances at 450 nm of serum diluted 1/15,625 with respect to serum obtained from control animals immunized with CFA alone.

sponse to MBP is antigen specific, as shown in Figure 3. Specifically, the proliferative response to a purified protein derivative of *Mycobacterium tuberculosis*, which occurs as a result of immunization with CFA, is not suppressed by feeding MBP. Feeding an irrelevant antigen such as BSA also does not affect the proliferative response to this protein derivative and only slightly suppresses the proliferative response to MBP. Finally, feeding MBP does not affect the proliferative response to BSA in animals immunized to BSA.

The effect of feeding MBP on the production of antibody to MBP was also examined. Rats fed MBP were immunized with MBP in CFA and bled 16 days after immunization. Levels of anti-MBP antibody in the serum were measured by enzyme-linked immunosorbent assay. When animals were fed at weekly intervals (days −14, −7, and 0), suppression of anti-MBP antibody response was only observed when rats were fed the highest (500 µg) dose tested (66% suppression, Fig. 4). Unlike the suppression of proliferative response, suppression of antibody production was not observed when rats were fed lower doses of MBP. When rats were fed antigen either before or after immunization under the other feeding schedule (days −7, −5, and −2 or +2, +5, and +7) the antibody response was not significantly affected, with only 15% suppression observed for both pre- and post-fed rats (data not shown).

Effect of feeding MBP fragments on EAE. It has been

reported that the encephalitogenic region of MBP for Lewis rats is a specific decapeptide sequence located at residues 75 to 84 of either guinea pig or bovine MBP, which by itself can induce EAE when injected into animals with CFA, whereas other regions of the molecule are nonencephalitogenic (23). Furthermore, for some antigens, including MBP, it has been reported that distinct suppressor determinants exist at sites different from immunogenic determinants (33, 34). We therefore investigated whether either encephalitogenic or nonencephalitogenic regions of MBP could by themselves prevent EAE via oral administration. Fragments of guinea pig MBP were generated by limited pepsin digestion and separated by column chromatography. The three different fragments generated by this procedure were fed separately to rats, and then animals were immunized with whole guinea pig MBP. It was found that both the disease-inducing (fragment 44–89) and nonencephalitogenic (fragments 1–37 and 90–170) fragments suppressed EAE when fed to rats, the nonencephalitogenic fragments being more effective in suppressing the disease than the encephalitogenic fragment (Table III). Furthermore, oral administration of both the encephalitogenic fragment (fragment 44–89) and the nonencephalitogenic fragment (fragment 1–37) suppressed subsequent proliferative response to whole MBP (Table III).

In terms of bovine MBP, it has been reported that a decapeptide, termed S79, which differs from the 75–84 encephalitogenic decapeptide by a single amino acid substitution (Gly for Asn) at the carboxyl terminus, suppresses EAE when injected into Lewis rats with CFA (28). We thus synthesized both the bovine encephalitogenic decapeptide and S79 decapeptide and fed them to animals before immunization with whole guinea pig MBP. The S79 decapeptide suppressed EAE and appears to be more suppressive of disease than the encephalitogenic decapeptide itself (Table III). Whole bovine MBP, which is not encephalitogenic in rats at doses encephalitogenic for guinea pig MBP (29), completely suppressed disease when fed to animals at the same dose as guinea pig MBP before immunization with guinea pig MBP (Table III).

DISCUSSION

Our results demonstrate, first, that oral administration of MBP is an effective means by which acute monophasic EAE in rats can be suppressed in an antigen-specific manner. Orally induced suppression is dose-dependent, with both clinical and histologic symptoms of disease being dramatically reduced. Second, feeding MBP to rats profoundly suppresses T cell proliferative response to MBP and, to a lesser extent, anti-MBP antibody production, which is consistent with orally induced suppression of immune responses for other antigens (8). Third, nonencephalitogenic fragments of MBP (peptides 1–37 and 90–170) are as effective in orally suppressing EAE as the intact protein. Thus, oral suppression of disease does not require the disease-inducing antigenic determinant of MBP.

The oral administration of MBP was most effective in suppressing EAE when administered before immunization, suggesting that the afferent limb of disease induction was affected most by feeding. Nonetheless, feeding after the induction of disease (starting as late as day +7) was also effective in at least partially suppressing disease, which indicates that later stages of the immune response following disease induction may also be susceptible to oral suppression. Studies are currently in progress to determine the effectiveness of orally administered MBP in suppressing adoptively transferred and chronic relapsing EAE.

Although feeding MBP after EAE induction did not completely suppress disease (60% suppression vs 100% suppression if animals were fed before immunization), it was as effective as feeding before immunization in suppressing the proliferative response to MBP (90% suppression). Different doses of orally administered MBP were required to suppress disease, proliferative responses, and antibody production, and, in the case of the antibody response, the interval of feedings influenced the suppressive effect. This suggests that different mechanisms may be involved in the induction and maintenance of oral tolerance for cell-mediated and humoral responses (30, 31) and for disease protection.

We have shown that orally administered fragments of MBP that do not contain the encephalitogenic sequence prevent disease and suppress proliferative responses to MBP, suggesting that suppressor determinants exist in the MBP molecule distinct from the encephalitogenic region and that these determinants can induce tolerance when dissociated from the disease-inducing determinant. Furthermore, our data suggest that nonencephalitogenic determinants may be more potent suppressors of disease than encephalitogenic determinants. Suppressor determinants that are distinct from antigenic determinants have been described in

TABLE III

Effect of feeding encephalitogenic and nonencephalitogenic fragments and peptides on the development of EAE

Feeding (μg)[a]	Incidence of EAE[b]	Proliferative Response[c]
Controls	19/25	7.0
Guinea pig MBP		
Fragment 1–37 (109)	0/9***	1.3
Fragment 44–89 (135)	3/11**	2.5
Fragment 90–170 (235)	0/4**	ND
Bovine MBP		
Whole (500)	0/10***	ND
Decapeptide S79 (30)	1/8***	ND
Encephalitogenic decapeptide (30)	4/8	ND

[a] Lewis rats were fed the indicated amounts of peptides or proteins (equimolar to 500 μg of whole guinea pig MBP) on days −7, −5, and −2 and immunized on day 0 with 50 μg of guinea pig MBP with CFA. Shown are the number of diseased rats of the total number tested. Clinical disease in controls and fed animals consisted of hind limb paralysis and incontinence.

[b] Groups were compared with immunized controls by χ^2 analysis. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

[c] Proliferative response of lymph node cells to whole MBP is expressed as stimulation index (experimental cpm/control cpm). ND, not determined.

hen's egg white lysozyme and appear in the amino terminus of the molecule (32, 33). Using the same EAE system we have studied (guinea pig MBP in Lewis rats), Chou et al. (34) reported that the encephalitogenic region of the MBP molecule was necessary for suppression of EAE when given with incomplete Freund's adjuvant (IFA). Driscoll et al. (35) reported similar results with the guinea pig. Swanborg (36), on the other hand, using guinea pig MBP in guinea pigs reported that an inhibitory determinant was present in fragment 43–89, which is distant from the encephalitogenic site (residues 114 to 121) and which suppressed disease when administered with IFA. The disparity between our results and those of Chou et al. (34) may relate to the differences in routes of administration of antigen used to induce suppression. The oral route may provide unique conditions that maximize the induction of suppression. For example, gut processing of antigen may modify and "biologically filter" antigen (37) in a manner that cannot be reproduced by other means, such as immunization with IFA or direct i.v. injection. In addition, the gut may provide specialized antigen-presenting cells, either in the intestinal epithelium (38) or Peyer's patch (39), which have been reported to be involved in activating suppressor inducer T cells. Studies are currently in progress to define the minimal amino acid sequences on the nonencephalitogenic determinants required to induce oral tolerance to EAE.

Orally induced tolerance is a normal immune response that is considered to function in the prevention of allergic and autoimmune reactions to food antigens (8). Although the oral administration of antigen has been widely studied as a means of suppressing the immune response for a number of different cellular (14, 15), protein (12, 13, 16, 17), and nonprotein (e.g., contact-sensitizing) antigens (11, 19), it has not been applied in the suppression of an autoimmune disease to a defined antigen until recently. We are aware of one published report in which the oral administration of type II collagen was used to suppress collagen-induced arthritis in mice (40) and of a preliminary report of EAE suppression by oral administration of MBP, in which 20 mg of MBP was fed and 58% of animals were protected from clinical disease (41). In the study of arthritis, collagen-induced arthritis was suppressed by feeding undenatured but not denatured type II collagen. There was a slight decrease in IgG2b anticollagen antibodies reported; T cell responses were not determined.

The specific immune mechanism by which oral administration of MBP or its fragments suppresses EAE is at the present time undefined. Adoptive transfer studies with animals fed other antigens (11–14, 17–19) have often shown that antigen-specific suppressor T cells are generated by feeding and are involved in actively suppressing the immune response. Other mechanisms, such as the production of soluble factors in the serum (20) and the formation of antigen-antibody complexes (21), have also been proposed and may represent additional or alternative mechanisms. Suppressor T cells have been shown to play a role in the modulation of EAE, being involved in the recovery from disease in rats (42, 43) and in the natural unresponsiveness to the disease in certain strains of mice (44). It is therefore possible that MBP-specific suppressor T cells are induced by orally administered MBP. Studies are presently in progress to determine the role of suppressor T cells in generating oral suppression of EAE via adoptive transfer of orally induced suppression from fed donors to naive recipients.

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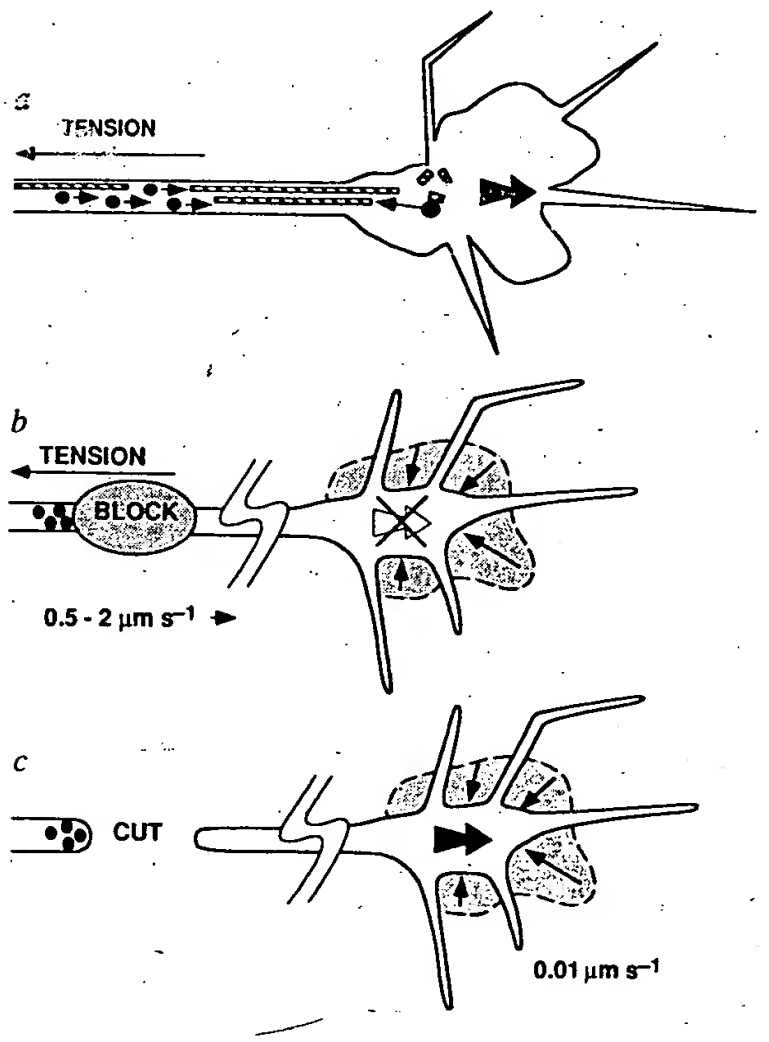

FIG. 4 Summary of growth cone behaviour. *a*, Factors carried by the fast transport system (filled circles) arrive at the growth cone and facilitate the elongation of the cytoskeletal network (hatched rectangles), allowing advance (solid arrow) to occur. *b*, When growth cones are deprived of fast transport material but remain attached to the axonal cytoskeleton, advance is no longer possible (crossed-out arrow) and growth cones collapse. These changes are propagated to the growth cone at the indicated rate of 0.5–2 μm s^{-1}. *c*, When growth cones are transected from the axonal cytoskeletal network and deprived of fast transport material, they collapse but are capable of advancing at the rate indicated. Thus, the only limitation to the forward movement of blocked growth cones is anchorage to the cytoskeleton.

than similarly irradiated non-severed growth cones (Fig. 3*c*). As both blocked and transected growth cones are deprived of fast transport vesicles to the same extent and have received equivalent doses of laser irradiation, cytoskeletal linkage to the cell body may account for the variance in motile behaviour. The possibility of a slow wave of Ca^{2+} influx propagating down the axon was ruled out as such waves in other systems are much more rapid[9]. Similarly, changes propagated as a result of a blockage of the slow transport system might be expected to travel at the slow transport rate of 0.01–0.05 μm s^{-1}.

The simplest explanation for our findings is that the axonal cytoskeleton provides a mechanical anchor to forward migration (Fig. 4*b*). For the axonal cytoskeleton to elongate in the absence of a bulk sliding process[1,2], cytoskeletal assembly must occur near the growth cone. Fast axonal transport of cytoskeletal elements may occur[5] but it is also likely that cytoskeletal proteins are conveyed by slow axonal transport (reviewed in ref. 10) and assembly is locally regulated at the growth cone. Such assembly could require fast transport components that either coassemble with the cytoskeleton or are short-lived cofactors in the assembly process (Fig. 4*a*). Transection (Fig. 4*c*) would relieve the tension axons are under[11–13], allowing the growth cone to advance. □

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Spontaneous loss of T-cell tolerance to glutamic acid decarboxylase in murine insulin-dependent diabetes

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INSULIN-DEPENDENT diabetes mellitus (IDDM) in non-obese diabetic (NOD) mice results from the T-lymphocyte-mediated destruction of the insulin-producing pancreatic β-cells and serves as a model for human IDDM[1]. Whereas a number of autoantibodies are associated with IDDM[2], it is unclear when and to what β-cell antigens pathogenic T cells become activated during the disease process. We report here that a T-helper-1 (Th1) response to glutamate decarboxylase develops in NOD mice at the same time as the onset of insulitis. This response is initially limited to a confined region of glutamate decarboxylase, but later spreads intramolecularly to additional determinants. Subsequently, T-cell reactivity arises to other β-cell antigens, consistent with intermolecular diversification of the response. Prevention of the spontaneous anti-glutamate decarboxylase response, by tolerization of glutamate decarboxylase-reactive T cells, blocks the development of T-cell autoimmunity to other β-cell antigens, as well as insulitis and diabetes. Our data suggest that (1) glutamate decarboxylase is a key target antigen in the induction of murine IDDM; (2) autoimmunity to glutamate decarboxylase triggers T-cell responses to other β-cell antigens, and (3) spontaneous autoimmune disease can be prevented by tolerization to the initiating target antigen.

We tested NOD mice from birth to 28 weeks of age for T-cell reactivity to β-cell antigens that are targets of IDDM-associated

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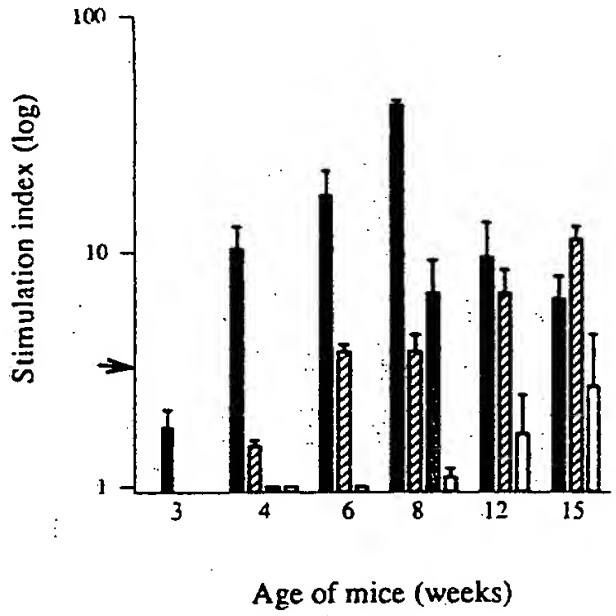

FIG. 1 Proliferative T-cell responses to β-cell antigens develop spontaneously in NOD mice in a defined chronological order. Antigen-induced blastogenesis was measured in spleen cells from newborn to 28-week-old female NOD mice (data from 3–15 weeks are shown). β-Cell antigens include GAD65 (black bars), Hsp65 peptide PALDSLTPANED[12] (striped bars), carboxypeptidase H (grey bar) and insulin (white bars). Data are expressed as stimulation indices (SI) ± standard error of the mean (s.e.m.), calculated from 3–5 mice tested individually in 2 separate experiments for each time point. Arrow indicates SI = 3, the level of significance. Carboxypeptidase H responses were only tested at 4 and 8 weeks. None of the control antigens (hen egg-white lysozyme, human serum albumin, *E. coli* β-galactosidase or murine myelin basic protein) induced T-cell proliferation at any age. Also, none of the β-cell antigens or control antigens induced proliferation of T cells from age-matched control BALB/c or (NOD × BALB/c) F_1 mice (data not shown).
METHODS. NOD (Taconic farms) and BALB/c mice (Jackson Laboratories) were housed under specific pathogen-free conditions. Spleen cells were tested directly *ex vivo* for proliferative recall responses. Cells were plated at 1×10^6 cells per well in 96-well microtitre plates in 200 μl HL-1 medium (Ventrex) containing 2 mM glutamine and 10 μg ml^{-1} antigen, or 7 μM peptide (the optimal concentrations for all time points tested) in triplicate. During the last 16 h of the 72 h culture period, 1 μCi [^{3}H]thymidine was added per well. Incorporation of label was measured by liquid scintillation counting. Human GAD65 (ref. 25) and *E. coli* β-galactosidase were both purified from recombinant bacteria using a hexahistidine tag and metal-affinity chromatography[26]. Bovine carboxypeptidase H was a generous gift from L. Fricker; human insulin was purchased from Eli Lilly.

autoantibodies. These included one of the two forms of glutamate decarboxylase[3,5] (GAD65, an early target of autoantibodies[6,9]), carboxypeptidase H[10], insulin[2] and the immunodominant determinant of heat-shock protein (Hsp65) (refs 11, 12).

Proliferative T-cell responses to these antigens developed spontaneously in a sequential order. First, a response to GAD arose at 4 weeks of age (Fig. 1), concurrent with the onset of insulitis. This reactivity increased during the next four weeks and then declined to background levels by week 16. At 6 weeks of age, responses to heat-shock protein appeared, increased until week 15 and then diminished (Fig. 1; also ref. 12). Similarly, whereas no response was detected to carboxypeptidase H at 4 weeks old, there was a strong anti-carboxypeptidase H response by week 8. In some mice, a weak response to insulin became detectable at 12–15 weeks old.

The spontaneous development of a proliferative T-cell response to GAD is consistent with, but does not prove, endogenous priming. We therefore tested GAD-reactive T cells for additional properties that distinguish activated/memory lymphocytes from resting/naive lymphocytes. First, we found that GAD-challenged freshly isolated T cells from 6–9-week-old NOD mice produce interferon-γ (IFN-γ), which is secreted only by preactivated Th1 lymphocytes[13] (Fig. 2*a*). Second, whereas the frequency of T cells reactive to control antigens constituted ~1 in 10^5 cells in the spleen of 6–9-week-old NOD mice, the frequency of GAD-reactive T cells was almost two orders of magnitude higher, which is consistent with clonal expansion (Fig. 2*a*). Third, GAD reactivity resided within the L-selectin$^-$ fraction of CD4$^+$ cells, a phenotype characteristic of activated lymphocytes[14] (Fig. 2*b*). These findings provide independent evidence that a potentially pathogenic[15] Th1-type T-cell population is spontaneously primed to GAD65 early in the development of NOD diabetes.

We mapped the fine specificity of the anti-GAD T-cell response using an overlapping set of peptides that span GAD65. The initial response, at 4 weeks, involved two adjacent peptides (amino acids 509–528 and 524–543, peptides 34 and 35, respectively; Fig. 3*a*). During the next 3 weeks, T-cell autoimmunity spread to several additional GAD determinants (including amino acids 246–266, peptide 17, which contains a region of sequence similarity with Coxsackie virus[9]; Fig. 3*b*, *c*). Subsequently, reactivity to GAD peptides declined (data not shown), paralleling the loss of response to the whole protein (Fig. 1).

The gradual diversification of the primed autoreactive T-cell repertoire in this naturally occurring autoimmune disease paral-

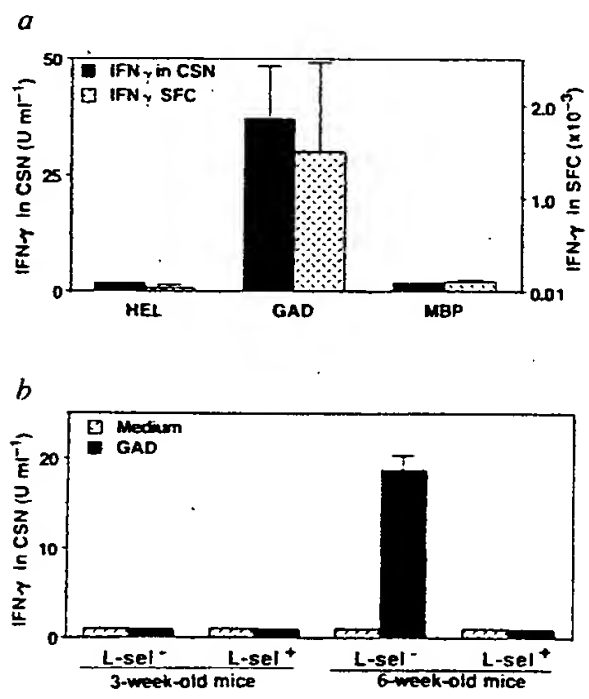

FIG. 2 GAD-specific T cells in 6–9-week-old female NOD mice are primed Th1 type CD4$^+$ lymphocytes based on their production of IFN-γ (*a* and *b*), enhanced clonal size (*a*) and L-selectin$^-$ phenotype (*b*). *a*, Detection of IFN-γ by ELISA in culture supernatants (CSN) of spleen cells from 6–9-week-old mice 48 h after challenge with GAD or control antigens hen egg-white lysozyme (HEL) and murine myelin basic protein (MBP). High concentrations of IFN-γ were detected only in cultures containing GAD. IFN-γ production was measured by ELISA[27] using IFN-γ-specific monoclonal antibodies R4-6A2 and XMG 1.2 (Pharmingen). T cells from age-matched BALB/c mice did not respond to GAD or to control antigens (data not shown). The frequency of antigen-specific IFN-γ-producing cells was determined by an ELISA spot technique[28] using the complementary IFN-γ mAbs. Frequency of antigen-induced spot-forming cells (SFC) is shown. Values are the mean ±s.e.m. from 5 female NOD mice, tested in triplicate cultures, with and without antigen. Results are from a single experiment and are representative of 3 experiments. *b*, Detection of GAD-specific, IFN-γ-producing cells in the L-selectin$^-$ (L-sel$^-$) subpopulation of CD4$^+$ cells. CD4$^+$ cells were isolated from the spleens by panning on goat–anti-mouse immunoglobulin (Zymed) and on anti-CD8 (mAb 58.6-72)-coated plates. CD4$^+$ cells were subfractionated using L-selectin-specific mAb MEL-14. CD4$^+$ L-selectin$^+$ and CD4$^+$ L-selectin$^-$ fractions were seeded in triplicate at 2×10^5 cells per well with irradiated (3,000 rad) spleen cells of 3-week-old NOD mice (5×10^5 cells per well) as a source of antigen-presenting cells. GAD-induced IFN-γ production was measured after 48 h by ELISA.

TABLE 1 Induced tolerance to GAD prevents the development of insulitis and the spread of T-cell autoimmunity

Treatment	Insulitis score	Spleen cell proliferation (SI ± s.e.m.)						
		β-galactosidase	GAD	GAD peptides			Hsp peptide	CPH
				No. 17	No. 34	No.35		
Uninjected	2.4 ± 0.2	1.0 ± 0.2	9.5 ± 2.1	4.8 ± 0.4	6.0 ± 0.1	2.9 ± 0.2	6.7 ± 1.0	ND
β-Galactosidase	2.6 ± 0.6	1.1 ± 0.1	15.4 ± 1.8	5.1 ± 0.6	5.1 ± 0.6	4.0 ± 0.2	6.6 ± 0.5	11.5 ± 0.9
GAD	0.1 ± 0.1	1.1 ± 0.03	1.6 ± 0.3	1.0 ± 0.05	1.2 ± 0.1	1.0 ± 0.1	1.2 ± 0.1	1.0 ± 0.02
Hsp-p	1.7 ± 0.4	1.1 ± 0.05	5.8 ± 0.2	4.5 ± 0.1	4.1 ± 0.3	4.2 ± 0.1	1.1 ± 0.04	4.4 ± 0.2
m-Hsp	1.8 ± 0.5	1.0 ± 0.1	4.2 ± 0.1	3.9 ± 0.1	3.9 ± 0.2	3.4 ± 0.2	1.0 ± 0.03	4.3 ± 0.2

Female NOD mice were injected intravenously at 3 weeks of age with 50 μg GAD65, β-galactosidase, mycobacterial Hsp65 (m-Hsp) or 0.1 μg immunodominant heat-shock peptide (Hsp-p) in PBS. At 12 weeks, mice were examined for insulitis and splenic T-cell responses to IDDM-associated autoantigens. Pancreatic tissue sections were stained using immunoperoxidase for insulin and counterstained with haematoxylin. Insulitis was scored blinded by examining 54 to 87 islets on 5 interrupted tissue sections from each pancreas. Severity of mononuclear cell infiltration was defined histologically: zero indicates lymphocytic infiltration; 1 indicates <25%; 2, 25–50%; 3, 50–75%; 4, >75%[23]. Proliferative splenic T-cell responses were measured as described for Fig. 1. Values are stimulation index (SI) ±s.e.m. Significant T-cell responses are underlined; a dashed underline is used for a borderline response. See Fig. 3 legend for GAD peptide numbering system. CPH, carboxypeptidase H. ND, not determined at this time point. Background counts in all groups was typically around 2,000 c.p.m. $N=8$ for the GAD-treated group and $N=5$ for all other groups.

lels similar findings in experimentally induced autoimmune disease[16,17]. Apparently lymphokine secretion by the first wave of autoreactive T cells in the target organ induces loss of T-cell tolerance to additional antigens, resulting in a cascade of autoimmune responses[18–20]. The activation of T cells reactive to additional β-cell antigens is likely to promote β-cell destruction (for example, Hsp-reactive $CD4^+$ T-cell clones can induce IDDM[11,12]).

GAD-reactive T cells were the first to arise among the autoantigens tested (Fig. 1). But did the anti-GAD response itself develop through intermolecular spreading, after a T-cell response to an unidentified β-cell antigen? If the development of T-cell reactivity to GAD is a primary event in the pathogenesis of IDDM, the inactivation of GAD-reactive T-cells before their spontaneous priming should prevent the cascade of T-cell responses to other β-cell antigens, insulitis and diabetes.

NOD mice were injected intravenously with GAD at 3 weeks of age, a treatment that causes unresponsiveness in antigen-reactive T cells[21]. Control groups received a similarly purified antigen (β-galactosidase), the immunodominant Hsp65 peptide, or mycobacterial heat-shock protein (which has been shown to vaccinate against murine IDDM[11]). The mice were examined for autoantigen-reactive T cells and insulitis at 12 weeks of age (Table 1), an age at which both reactive T cells and insulitis are clearly established in untreated NOD mice. Seventy five per cent of the GAD-treated mice (but none of the controls), displayed no T-cell reactivity to GAD, indicating complete tolerization. The GAD-tolerized mice showed no reactivity to other β-cell antigens and were completely free of insulitis (score zero). If there were another effector T-cell population in the islets, specific for an unknown β-cell antigen, that preceded the anti-GAD response, the release of cytokines by this population should have promoted T-cell responses to β-cell antigens and insulitis[19,20]. Twenty five per cent of the GAD-treated mice were not completely tolerized to GAD, as evidenced by weak residual GAD reactivity (stimulation index ~3) and displayed very limited peri-insulitis. In contrast, although tolerization to both of the heat-shock protein antigens was complete, these treatments reduced, but did not prevent, the development of T-cell responses to other β-cell antigens or insulitis—as would be expected if a secondary element were removed from the amplificatory cascade.

In ongoing experiments examining the effects of GAD tolerization on diabetes incidence, all of the GAD-treated mice ($n=17$; now 37 weeks old) have normal glucose levels, whereas 70% of the mice receiving control antigens developed hyperglycaemia by 19 weeks of age ($n=20$). Five GAD-treated mice were examined at 30 weeks of age for insulitis. Four pancreata were completely free of insulitis (score 0.0) and one showed very limited peri-insulitis (J.T. and D.L.K., unpublished results). Thus, GAD

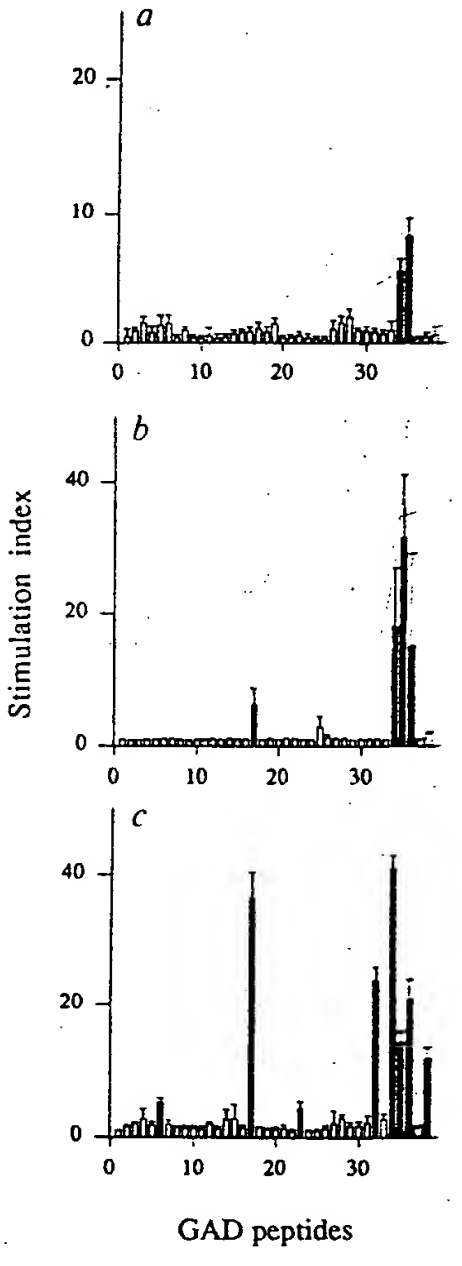

FIG. 3 Intramolecular spreading of autoimmunity within the GAD molecule. Spleen cells were tested from 4-week-old (*a*), 5-week-old (*b*) and 7-week-old (*c*) NOD mice for proliferative responses to GAD65 peptides. A set of 38 peptides, each 20–23 amino acids long, span the entire human GAD65 (ref. 25) molecule with overlaps of 5 amino acids. These peptides are numbered successively from the N terminus. Peptides that triggered stimulation indices >3 are indicated as black bars. These peptides did not induce proliferation in T cells from NOD mice <3 or >16 weeks in age (paralleling reactivity to whole GAD), or from control $(BALB/c \times NOD)F_1$ mice (data not shown). Data are represented as the mean SI ± s.e.m. calculated from 3–6 mice tested individually in two separate experiments for each age group. METHODS. Proliferation was assayed as described for Fig. 1. Peptides were present in cultures at 7 μM and label was added during the last 16 h of a 5-day culture. Peptides were synthesized using standard F-moc chemistry and purified by reverse-phase HPLC (Advanced Chem-tech). The sequences of stimulatory peptides are: peptide 509–528 (no. 34), (IPPSLRYLED–NEERMSRLSK); peptide 524–543 (no. 35), (SRLSKVAPVIKARMMEYGTT); and peptide 247–266 (no. 17), (NMYAMMIARFKMFPEVKEKG). Murine and human GAD65 share 95% amino-acid identity and are 98% conserved[29]. Underlined amino acids are conservatively substituted in murine GAD65. In separate experiments, the murine forms of these peptides were tested and produced similar results.

tolerization can prevent the development of clinical diabetes in NOD mice.

Prevention of diabetes in NOD mice has also been reported following oral feeding of insulin[22], immunization with complete Freund's adjuvant[23], heat-shock protein[11] and heat-shock protein-specific T cells[12]. These treatments are thought to work by induction of active protective immune responses[11,12,22,23] and have not been shown to prevent insulitis. In contrast, we have shown that in the absence of T-cell reactivity to GAD, autoimmunity to β-cells does not develop, demonstrating the pathogenic significance of this response.

Our data qualify GAD as a key antigen in the induction of murine IDDM. The initial spontaneous anti-GAD response leads to the recruitment of additional β-cell antigen-reactive T cells. This cascade of autoimmune responses can be circumvented by inactivating GAD-reactive T cells. As a similar autoimmune progression may also occur in human IDDM[24] and in other T-cell-mediated autoimmune diseases (involving different autoantigens), these findings should be useful in the design of immunotherapies. □

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Immune response to glutamic acid decarboxylase correlates with insulitis in non-obese diabetic mice

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KNOWING the autoantigen target(s) in an organ-specific autoimmune disease is essential to understanding its pathogenesis. Insulin-dependent diabetes mellitus (IDDM) is an autoimmune disease characterized by lymphocytic infiltration of the islets of Langerhans (insulitis) and destruction of insulin-secreting pancreatic β-cells[1]. Several β-cell proteins have been identified as autoantigens, but their importance in the diabetogenic process is not known[2]. The non-obese diabetic (NOD) mouse is a murine model for spontaneous IDDM[3]. Here we determine the temporal sequence of T-cell and antibody responses in NOD mice to a panel of five murine β-cell antigens and find that antibody and T-cell responses specific for the two isoforms of glutamic acid decarboxylase (GAD) are first detected in 4-week-old NOD mice. This GAD-specific reactivity coincides with the earliest detectable response to an islet extract, and with the onset of insulitis. Furthermore, NOD mice receiving intrathymic injections of GAD65 exhibit markedly reduced T-cell proliferative responses to GAD and to the rest of the panel, in addition to remaining free of diabetes. These results indicate that the spontaneous response to β-cell antigens arises very early in life and that the anti-GAD immune response has a critical role in the disease process during this period.

We have cloned and expressed a panel of candidate murine β-cell autoantigens. This panel consists of: (1) the two isoforms of GAD, GAD65 and GAD67; (2) peripherin; (3) carboxypeptidase H; and (4) heat-shock protein 60 (Hsp60). (GAD, an enzyme required for γ-aminobutyric acid synthesis, is recognized by T cells and antibodies in preclinical and recent-onset diabetics[4–6]; peripherin is a cytoskeletal protein which is recognized by antibodies in the serum of NOD mice[7]; carboxypeptidase H, expressed in β-cell secretory granules, is recognized by antibodies in the serum of preclinical diabetics[8]; and Hsp60 stimulates T-cell and antibody responses in NOD mice[9].) It is not known, however, whether any of these autoantigens has a critical role in the initial events in the diabetogenic process.

In NOD mice, β-cell destruction is T-cell-dependent[10–13]. We therefore examined T-cell responses against the panel of β-cell antigens in unimmunized NOD mice. T-cell proliferation in response to an islet extract was first detected in splenic cell cultures established from 4-week-old females (Fig. 1*b*). There was a concomitant and significant response to GAD65 and GAD67. T-cell responses for peripherin, carboxypeptidase H and Hsp60 could first be seen in cultures established from 6-week-old NOD females (Fig. 1*c*). By 8 weeks of age, NOD mice showed enhanced responses to the entire panel (Fig. 1*d*). Furthermore, diabetic and 24-week-old non-diabetic NOD females also exhibited responses to the panel (Fig. 1*e*,*f*). In contrast, no response at any age could be detected with amylin, a 37-amino-acid polypeptide found in the insulin secretory granules of β-cells[14] which binds I-A^{g7} (data not shown). This suggests that not all β-cell antigens become targets for the diabetogenic response. Finally, the response to the panel of β-cell antigens and to the islet extract can be completely blocked in cultures established from 8-week-old NOD female mice by addition of the anti-CD4 antibody GK1.5 (data not shown). Similar treatment with the anti-CD8 antibody 53.7.3 had no effect on the responses. Therefore, we are measuring a $CD4^+$ T-cell proliferative response.

To examine further the *in vivo* effects of these T-cell responses, we measured antibodies specific for the panel of β-cell antigens. No response to the panel could be detected in sera from animals of 3 weeks old using an enzyme-linked immunoassay (Fig. 2),

Modulating autoimmune responses to GAD inhibits disease progression and prolongs islet graft survival in diabetes-prone mice

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In nonobese diabetic (NOD) mice, β-cell reactive T-helper type 1 (Th1) responses develop spontaneously and gradually spread, creating a cascade of responses that ultimately destroys the β-cells. The diversity of the autoreactive T-cell repertoire creates a major obstacle to the development of therapeutics. We show that even in the presence of established Th1 responses, it is possible to induce autoantigen-specific anti-inflammatory Th2 responses. Immune deviation of T-cell responses to the β-cell autoantigen glutamate decarboxylase (GAD65), induced an active form of self-tolerance that was associated with an inhibition of disease progression in prediabetic mice and prolonged survival of syngeneic islet grafts in diabetic NOD mice. Thus, modulation of autoantigen-specific Th1/Th2 balances may provide a minimally invasive means of downregulating established pathogenic autoimmune responses.

Insulin-dependent diabetes mellitus (IDDM) results from the T cell-mediated destruction of the insulin-producing pancreatic β-cells (reviewed in ref. 1–3). IDDM begins with an asymptomatic stage during which the β-cells are gradually destroyed. We have shown in an animal model of human IDDM, the nonobese diabetic (NOD) mouse, that a T helper 1 (Th1) response to glutamic acid decarboxylase (GAD65) arises at 4 weeks of age, concurrent with the onset of insulitis[4]. Subsequently, T-cell autoimmunity spreads to other β-cell antigens in a cascade of responses that ultimately lead to IDDM (ref. 4, 5).

In prediabetic humans, circulating β-cell autoantibodies provide markers of the ongoing autoimmune process, often years before disease onset[6,7]. However, individuals determined to be at risk for IDDM are likely to have an established diverse autoreactive T-cell repertoire. At this stage, it is not clear how to control the diverse repertoire of committed autoreactive T cells and to inhibit disease progression. Although immunosuppressants, cytokines and antibodies against T cells can inhibit disease onset in prediabetic rodents[1,3], such treatments lack specificity and may debilitate immune system function. Immunotherapeutics directed at blocking T-cell receptor–MHC (major histocompatibility complex) interactions can be highly specific[8,9]; however, these approaches may be confounded by the complexity of the autoreactive T-cell population and the genetic diversity of MHCs within the patient population. We therefore tested whether an alternative strategy, designed to induce antigen-specific anti-inflammatory Th2 responses[10–13], could deter disease development. We show that well after the establishment of autoimmunity, administering GAD65 to NOD mice in a mode that promotes Th2 responses, can shift the GAD65-specific Th1/Th2 balance and can inhibit disease progression.

Based on the ability of GAD65 administration to protect β-cells in prediabetic NOD mice with established autoimmunity, we examined whether this treatment could also aid in the reversal of IDDM by protecting syngeneic islet grafts in diabetic NOD mice. Usually, transplanted syngeneic β-cells are rapidly destroyed in diabetic NOD mice by the same autoimmune mechanism that destroyed the original islets[14,15]. We found that pretreating recipient NOD mice with GAD65 prolonged syngeneic islet graft survival without the use of immunosuppressants. Thus, autoantigen administration in modes designed to promote anti-inflammatory T-cell responses may provide minimally invasive therapies to aid the prevention and reversal of IDDM.

Antigen administration promotes a IgG1 response

Injection of antigen in incomplete Freund's adjuvant (IFA) has been recently shown to strongly stimulate antigen-specific Th2 responses[16]. However, NOD mice have been reported to have deficiencies in Th2 development[17], and (after 4 weeks of age) have an established Th1 response to GAD65 (ref. 4). Therefore, it was unknown whether it was possible to modulate GAD65-specific Th1/Th2 responses in prediabetic NOD mice and whether this could interfere with disease progression.

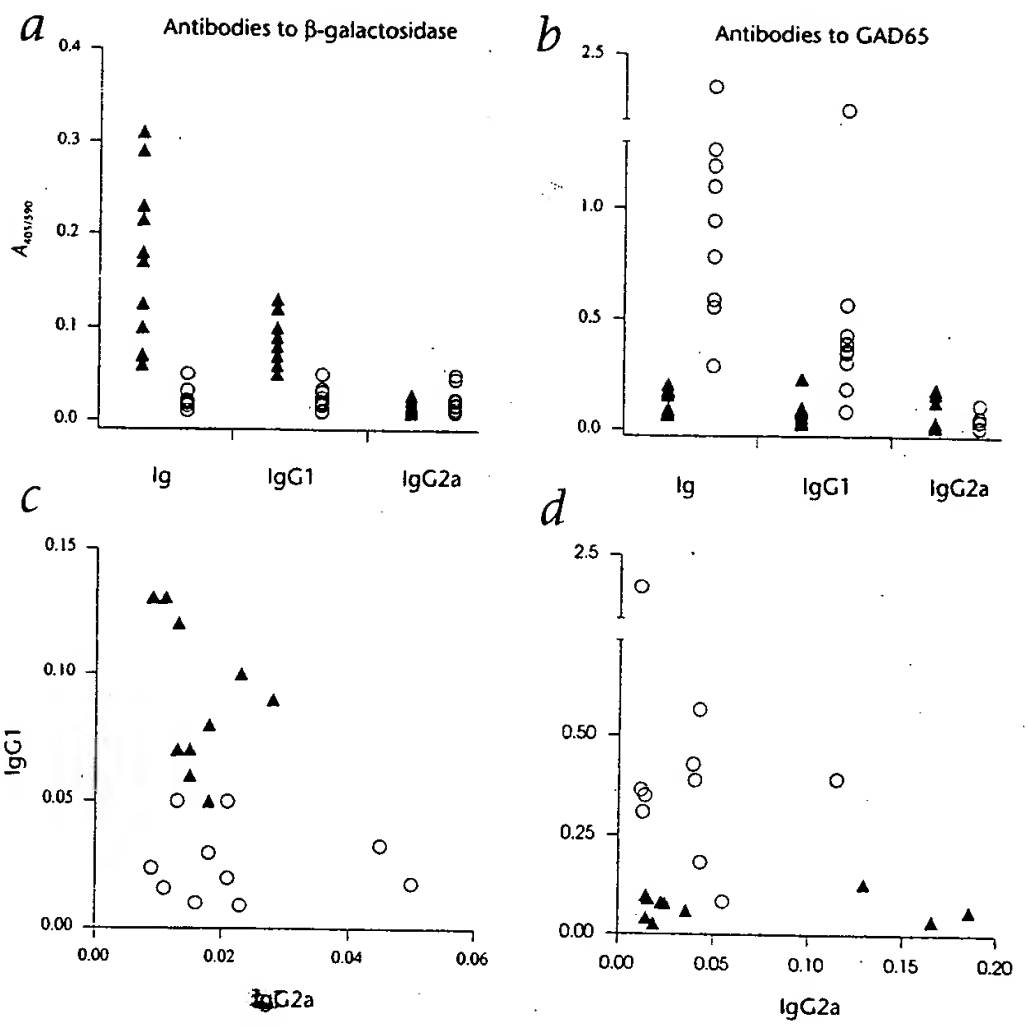

Fig. 1 Antigen administration induces antigen-specific IgG1 antibodies. Animals were treated with β-galactosidase (▲) or GAD65 (○), and antibodies to β-galactosidase (*a* and *c*) and GAD65 (*b* and *d*) were characterized using isotype-specific ELISA assays. *c* and *d*, IgG1 vs. IgG2a antibody levels for individual mice. The level of GAD65 autoantibodies in the β-galactosidase-treated group was similar to that found in unmanipulated NOD mice (data not shown). Serial dilutions of sera showed a linear relationship with resulting absorbance measures. The background absorbance ranged between 0.06 and 0.1. The data are represented as the mean absorbance values over background of triplicate samples from individual mice (n = 10 for each group). Experimental and control serum samples were tested simultaneously in two separate assays. Antibodies to GAD65 or β-galactosidase in sera from untreated BALB/c and AKR mice were at background levels.

We treated NOD mice only once with GAD65 or control β-galactosidase in IFA at 8 weeks of age, well after the onset of insulitis. Four weeks later, anti-β-galactosidase immunoglobulins were readily detected in serum samples from the β-galactosidase-treated group, but were only slightly above background levels in sera from the GAD65-treated group (Fig. 1*a*). High levels of GAD65 autoantibodies were present in almost all serum samples from the GAD65-treated group, whereas mice injected with β-galactosidase had low levels of GAD65 autoantibodies (Fig. 1*b*), as did unmanipulated NOD mice (data not shown). The induced β-galactosidase and GAD65 antibodies were predominantly IgG1 (Fig. 1), indicating these treatments primed antigen-specific Th2 help[10]. In individual mice, there tended to be an inverse correlation between IgG1 and IgG2a GAD65 antibody levels (Fig. 1*d*).

Antigen administration induces a Th2 response

Traditionally, it has been difficult to define Th1 and Th2 activity because of the low precursor frequency of antigen-specific T cells. We used an improved ELISPOT assay[16] to monitor frequency of splenic antigen-specific T cells that produce Th1- and Th2-type cytokines following the administration of control β-galactosidase or GAD65. We observed that β-galactosidase administration predominantly primed an increase in the frequency of interleukin-4 (IL-4) and IL-5 secreting β-galactosidase-specific Th2 cells (Fig. 2*a*). As expected, β-galactosidase-reactive T cells were infrequently observed in the GAD65-treated group.

Interferon-γ (IFN-γ)-secreting GAD65-specific Th1 cells were frequently detected in β-galactosidase-treated mice (as previously described in unmanipulated NOD mice[4]) whereas IL-4- and IL-5-secreting GAD65-specific Th2 cells were rarely detected (Fig. 2*b*). Following a single treatment with GAD65, there was a dramatic increase in the frequency of IL-4- and IL-5-secreting GAD65-reactive T cells, which on average were 15-

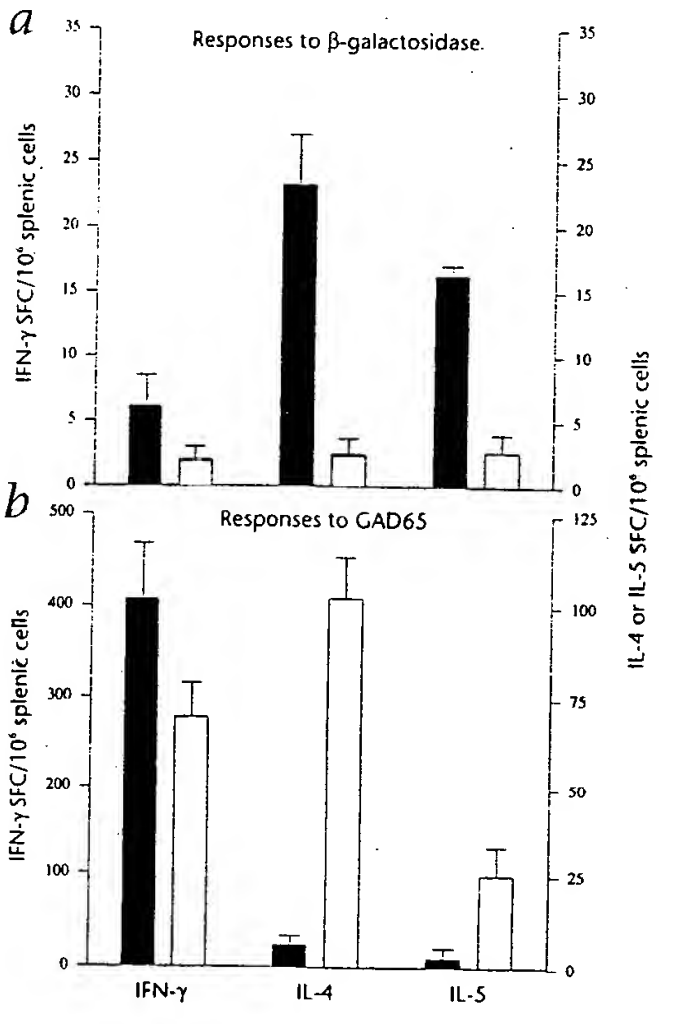

Fig. 2 Antigen administration stimulates the expansion of antigen-specific Th2 cells. The frequency of (*a*) β-galactosidase and (*b*) GAD65-specific IFN-γ, IL-4- and IL-5-secreting splenic T cells in β-galactosidase (■) and GAD65 (□)-treated mice was determined by ELISPOT. Data are represented as the mean number of spot-forming colonies (SFC) per 10^6 splenic T cells ± s.e.m. Samples were tested in triplicate. Experimental and control mice were tested simultaneously in two separate experiments (n = 5 for each group).

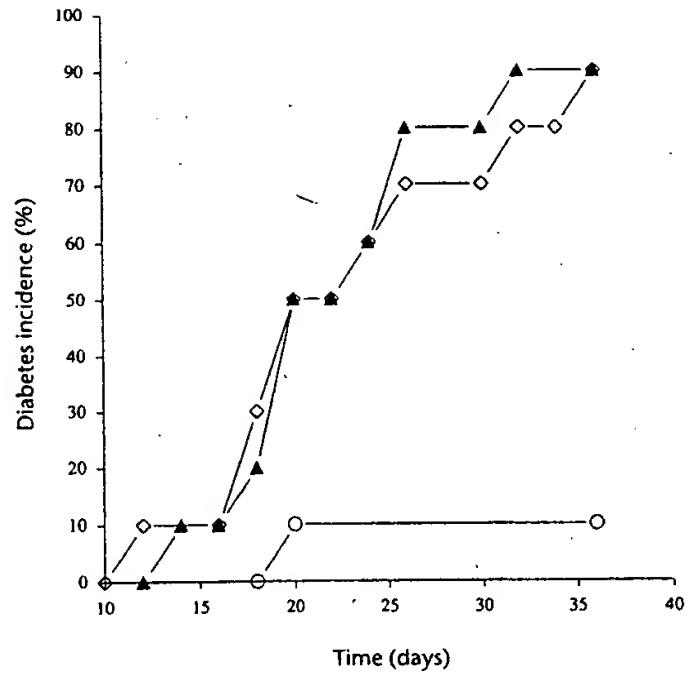

Fig. 3 Adoptive cotransfer of splenic T cells from GAD65-treated mice protects recipient mice from IDDM. Splenic mononuclear cells from mice treated with β-galactosidase (▲) or GAD65 (○) were cotransferred with T cells from diabetic NOD mice to 5-week-old irradiated female NOD mice. A positive control group received cells only from untreated-diabetic NOD mice (◇). Blood glucose levels were monitored frequently, and animals were considered diabetic after two consecutive blood glucose levels of >13 mmol/l. (n = 10 for all groups.)

and 10-fold as frequent (respectively) as in the control group (Fig. 2*b*). Furthermore, the average frequency of GAD65-specific IFN-γ-secreting T cells in GAD65-treated mice was 68% of that found in the control group, suggesting that this treatment limited the expansion of Th1 responses to GAD65. Thus, both β-galactosidase and GAD65 administration induced antigen-specific Th2 responses in recipient mice.

GAD65 treatment prevents adoptive transfer of IDDM

To examine whether the induced T-cell responses could inhibit an established pathogenic autoimmune response, we tested the ability of T cells from β-galactosidase- and GAD65-treated NOD mice to inhibit the adoptive transfer of diabetes. We observed that 90% of the mice receiving a mixture of splenic mononuclear cells from β-galactosidase and diabetic mice developed IDDM within 5 weeks after transfer — similar to a positive control group that received mononuclear cells exclusively from diabetic mice (Fig. 3). However, only 10% of the mice that received a mixture of mononuclear cells from GAD65-treated and diabetic mice developed IDDM ($P < 0.01$). Thus, GAD65 (but not β-galactosidase) treatment induces potent regulatory cells in prediabetic NOD mice that are capable of blocking target tissue destruction by a diverse, activated effector T-cell population.

GAD65 administration inhibits proliferative T-cell responses

We tested splenic T cells for proliferative responses to GAD65 and the immunodominant peptide of heat shock protein (hsp65, peptide 277, ref. 18) 4 weeks after treatment. Splenic T cells from both mice treated with IFA (alone) and those treated with β-galactosidase displayed strong T-cell responses to GAD65 and hsp65 (Table 1), which were similar in magnitude to the responses by splenic T cells from age-matched unmanipulated NOD mice[4]. In contrast, NOD mice injected with GAD65 displayed markedly reduced proliferative T-cell responses to GAD65 (Table 1). As ELISPOT analysis revealed that the frequency of GAD65-reactive Th1 cells was reduced by only 32% in GAD65-treated mice (Fig. 2*b*), these data suggest that the primed GAD65-specific Th2 response actively downregulated Th1 proliferation *in vitro*. However, responses to control concanavalin A (conA) and recall responses to hen egg white lysozyme (HEL) were unaffected by GAD or β-galactosidase treatment (Table 1). It is interesting that the inhibitory effect was not confined only to the administered autoantigen, as proliferative responses to hsp65 were also reduced in GAD65-treated mice. Indeed, the frequency of hsp65-reactive Th1 cells, as determined by ELISPOT analysis, was reduced by 30% in GAD65-treated prediabetic NOD mice (data not shown), suggesting that the induction of GAD65-specific Th2 responses limited the inflammatory cascade of β-cell reactive T-cell responses.

GAD65 treatment inhibits disease progression

Examination of the pancreases from 12-week-old control mice treated with IFA (alone) or β-galactosidase revealed that all islets had infiltrating lymphocytes. The severity of insulitis in control animals (Table 1) was similar to that observed in unmanipulated NOD mice[4]. In contrast, in the GAD65-treated group,

Table 1 GAD65 administration inhibits proliferative T-cell responses to β-cell autoantigens and insulitis

Treatment	Spleen cells					Lymph node cells		Insulitis score
	β-gal.	GAD65	hsp65	HEL	conA	HEL	PPD	
IFA alone	1.1 ± 0.2	10.9 ± 2.6	10.9 ± 1.9	1.0 ± 0.2	18.6 ± 2.1	47.8 ± 3.4	52.4 ± 4.7	2.6 ± 0.7
β-gal.	4.1 ± 1.9	13.1 ± 2.9	11.5 ± 3.4	1.1 ± 0.1	20.3 ± 1.4	50.3 ± 2.9	50.1 ± 3.6	2.1 ± 1.6
GAD65	1.3 ± 0.2	4.2 ± 0.9	7.3 ± 2.5	0.9 ± 0.3	18.1 ± 1.2	48.7 ± 4.3	56.7 ± 3.1	1.3 ± 1.3

The mean antigen-induced T-cell proliferation over background was expressed as stimulation index ± s.e.m. (n = 6 for each group). Primed lymph node T-cell recall responses to HEL or purified protein derivative (PPD) were unaffected by treatment with GAD65 or β-galactosidase. The background for medium alone ranged from 1300 to 2800 c.p.m. Mice from control and experimental groups were tested simultaneously in two separate experiments (using triplicate cultures). None of the antigens induced significant proliferation of splenic T cells from BALB/c mice (data not shown). Insulitis score was determined as previously described[4,29]. (n = 6 for each group.)

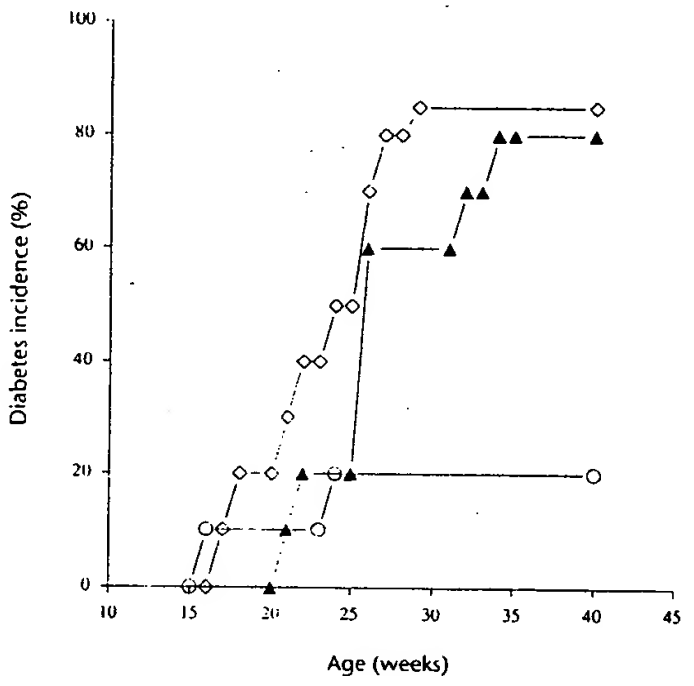

Fig. 4 Administration of GAD65 after the establishment of β-cell autoimmunity inhibits disease progression. Female NOD mice were treated at 8 weeks of age with IFA alone (◇), β-galactosidase (▲), or GAD65 (○). The mice were followed for the onset of hyperglycemia. (n = 10 for each group.)

40% of the islets were free of lymphocytic infiltrates and insulitis was less severe. Because the treatments were begun well after the onset of insulitis, the presence of some lymphocytic infiltrates in GAD65-treated mice was not unexpected. Thus, administering GAD65 to prediabetic mice inhibited lymphocytic infiltration of the islets.

Approximately 80% of the control mice developed IDDM by 35 weeks of age (Fig. 4), paralleling the disease course observed in unmanipulated female NOD mice. In contrast, 80% of the GAD65-treated mice showed no signs of hyperglycemia at 40 weeks of age ($P < 0.03$). Protection from IDDM has also been reported following administering GAD67 (ref. 19) and GAD65 (before the establishment of autoimmunity)[20] in IFA. We detected high levels of GAD65 IgG1 autoantibodies in the sera of all 40-week-old GAD65-treated mice that remained disease-free. However, the two GAD65-treated mice that developed IDDM had very low levels of GAD65 autoantibodies at the time of disease onset (data not shown).

GAD65 treatment prolongs syngeneic islet graft survival

We next examined whether this therapy could be extended to protect transplanted islets from established autoimmune responses in diabetic NOD mice. Immediately following the onset of diabetes, mice were maintained on insulin and were treated with control antigen, or with one of the following β-cell autoantigens; GAD65, hsp65 (peptide 277), or the B-chain of insulin (which contains the immunodominant determinant[21,22]). Following transplantation of newborn islets, exogenous insulin administration was discontinued and no immunosuppressants were used to block rejection. We observed that mice treated with the control antigen, the hsp peptide or insulin B-chain became diabetic approximately 10 days post transplantation (Fig. 5). In contrast, GAD65-treated mice remained euglycemic for an average of 48 days post transplantation. The GAD65-treated mice had residual islet cell function, which allowed them to survive in a chronic hyperglycemic state up to 20 weeks post transplantation (data not shown).

Discussion

Inhibiting autoimmune disease progression. IDDM is thought to be mediated by proinflammatory Th1 type cells. During the disease process, autoantigen recognition spreads intermolecularly and intramolecularly, creating a major obstacle for the design of immunotherapies. We tested whether at advanced stages of the disease process, the diverse autoreactive Th1 cell pool could be downregulated by the induction of an anti-inflammatory Th2 response to a single antigen. As prediabetic NOD mice have established Th1 responses to GAD65 (ref. 4), as well as deficiencies in Th2 development[17], it was not known whether GAD65 administration would promote pathogenic or regulatory T-cell responses.

We observed that a single administration of GAD65 in IFA to prediabetic NOD mice boosted Th2-dependent IgG1 GAD65 antibodies. ELISPOT analysis revealed this treatment promoted the expansion of IL-4- and IL-5-secreting GAD65-reactive T cells (confirming the activation a GAD65-specific Th2 response) and limited the expansion of IFN-γ secreting GAD65-specific T cells. Thus, even after the establishment of Th1 autoimmunity, it is possible to additionally engage Th2 cells and dramatically alter the balance of antigen-specific Th1/Th2 cells. We favor the interpretation that GAD65 administration guided not fully differentiated or uncommitted antigen-specific Th0 cells toward a Th2 lineage, rather than altered the phenotype of activated GAD65-specific Th1 cells.

GAD65 administration markedly reduced proliferative T-cell responses to other β-cell autoantigens, the frequency of hsp65-reactive T cells and lymphocytic infiltration in the islets. Consistent with the induction of an active tolerance mechanism, splenic T cells from GAD65-treated mice inhibited the adoptive transfer of disease. Finally, GAD65 administration significantly reduced long-term IDDM incidence. Collectively, these data show that GAD65 administration, even after the establishment of Th1 responses, can induce a Th2 response, which shifts the Th1/Th2

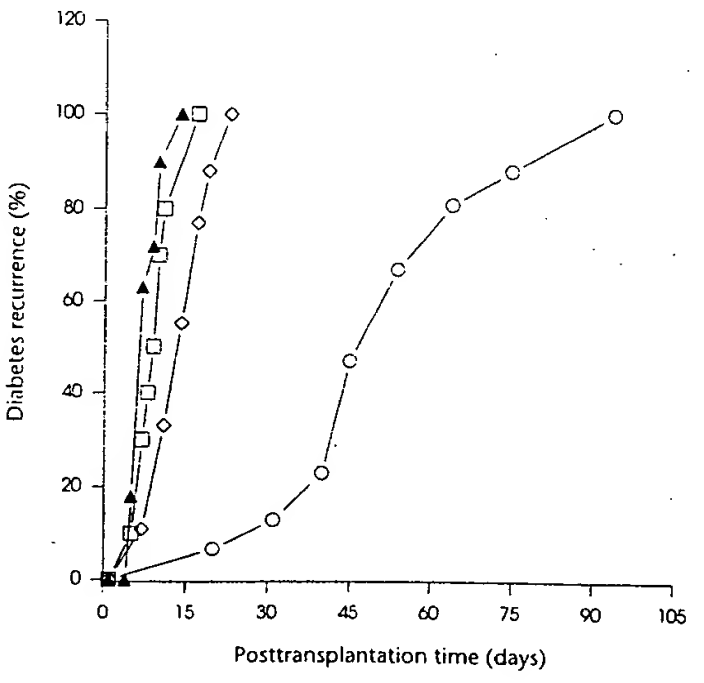

Fig. 5 Administration of GAD65 prolongs survival of syngeneic islet grafts in diabetic NOD mice. Islets were transplanted into diabetic NOD mice that had been treated with β-galactosidase (▲), GAD65 (○), insulin B-chain (□), or hsp65 peptide (◇), and insulin administration was discontinued. Data are presented as time post transplantation at which hyperglycemia recurred (blood glucose levels >13 mmol/l). (n = 14 for β-galactosidase, n = 17 for GAD65, n = 9 for insulin B-chain and n = 11 for hsp65.)

balance and is associated with inhibition of disease progression.

Nonspecific immunostimulation (for example, from viral infection) can reduce diabetes incidence in NOD mice[1-3]. However, although β-galactosidase administration induced antigen-specific Th2 responses, these responses were not associated with inhibition of disease progression. Only the priming of Th2 responses to a disease-associated autoantigen (GAD65) induced an active form of β-cell tolerance, presumably by targeting an anti-inflammatory response to the islets. A similar immune deviation process is likely to underlie the protective effects of administering other β-cells autoantigens in IFA (ref. 18–22).

In humans, an inverse relation between humoral and proliferative T-cell responses to GAD has been reported in individuals at risk for IDDM, and high autoantibody titers to GAD have been associated with slower disease progression[23,24]. We observed that GAD65 autoantibodies remained at high levels in GAD65-treated NOD mice that did not develop IDDM, but were at low levels in the few GAD65-treated NOD mice that developed disease, consistent with GAD65-specific Th2 regulation of disease progression (see also ref. 25). These findings underscore the possibility that the type of T-cell response to GAD65 may be a crucial factor in determining the course of the disease. Our data also suggest that changes in the pattern of antigen-specific T-cell responses and autoantibody isotypes may provide markers to monitor the efficacy of immunotherapeutics.

Reversal of IDDM by islet transplantation. Reversal of IDDM by islet transplantation requires the capability of inhibiting both the alloresponse to the graft and the autoimmune response that initially destroyed the recipient's β-cells. Current strategies to prolong graft survival rely on depletion of autoreactive T cells and immunosuppressive drugs[14,15]. Based on the ability of GAD65 treatment to prevent the destruction of β-cells in prediabetic NOD mice, we tested the ability of this treatment to protect transplanted syngeneic β-cells from the established autoimmune responses in diabetic NOD mice. Administering GAD65 to recipient diabetic NOD mice before transplantation greatly extended syngeneic islet graft survival. Immunosuppressants were not used, demonstrating that GAD65 treatment alone can downregulate established autoimmune responses. Although insulin B-chain and hsp65 peptide administration did not prolong islet graft survival, these treatments have been shown to inhibit disease onset in prediabetic NOD mice[18,21,22]. We have observed that administration of insulin in IFA induces antigen-specific Th2 responses in prediabetic mice, but only one-fourth as many as treatment with GAD65 (J.T., unpublished observations). This insulin-specific Th2 response may underlie the protective effects of insulin administration in prediabetic NOD mice, but may be insufficient to inhibit graft rejection in diabetic NOD mice. Although these studies did not address alloresponses, they do show that autoimmune responses can be inhibited via GAD65 administration, which may help reduce reliance on immunosuppression in human islet transplantation protocols.

Conclusions. Our work, as well as that of others, demonstrates that autoantigen-based immunotherapies can activate immune responses that are capable of downregulating an established, diverse autoimmune response. This approach, in combination with adjuvants, cytokines or altered peptide ligands that help guide an antigen-specific immune response toward one that is anti-inflammatory, may provide minimally invasive therapies to aid the prevention and reversal of IDDM.

Methods

Mice. NOD mice were purchased from Taconic Farms (Germantown, New York) and bred under specific pathogen-free conditions. Only female NOD mice were used in this study. In our NOD mouse colony, insulitis begins at 4 weeks of age. The average age of disease onset is at 22 weeks, with about 80% of the mice displaying IDDM by 30 weeks of age.

Antigens. Mouse GAD65 (ref. 26) and control *Escherichia coli* β-galactosidase were purified as previously described[4]. The hsp65 immunodominant peptide[18] was synthesized by standard fluorenyl methyloxycarbonyl (Fmoc) chemistry and purified by chromatography. Control hen egg white lysozyme peptide HEL_{11-25}, immunogenic in NOD mice, was provided by E. Sercarz. The amino acid composition of each peptide was verified by mass spectrometry. Insulin B-chain was purchased from Sigma.

GAD65 autoantibody assays. At 8 weeks of age, NOD mice received a single intraperitoneal (i.p.) injection of 100 μg β-galactosidase or GAD65 in 50% IFA (Gibco BRL, Gaithersburg, Maryland). Four weeks later, serum samples were tested for GAD65 and β-galactosidase by ELISA. β-Galactosidase or GAD65 (Synectics Biotechnology, Stockholm) at 10 μg/ml was bound to 96-well plates (Nunc, Ruskilde, Denmark), in 0.1 M $NaHCO_3$, pH 9.6 (β-galactosidase) or pH 8.5 (GAD65) at 4 °C overnight. The wells were rinsed with PBS and then blocked with 3% BSA in PBS for 1 h. Mouse sera was added (0.1 ml of a 1/500 dilution) and incubated 1 h at 37 °C. Following washing, bound immunoglobulin was characterized using affinity-purified horseradish peroxidase (HRP)-coupled goat anti-mouse IgG+A+M (H+L) (Pierce, Rockford, Illinois), or HRP-coupled goat anti-mouse isotype specific antibodies for IgG1 and IgG2a (Southern Biotech Associates, Birmingham, Alabama) and 2,2'-azino-*bis*(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS). Serum samples from untreated BALB/c and AKR mice were used as negative controls.

ELISPOT analysis. At 8 weeks of age, mice received a single i.p. injection of 100 μg β-galactosidase or GAD65 in 50% IFA. Fourteen days later, splenic T cells were isolated and the frequency of β-galactosidase and GAD65-specific T cells secreting IL-4, IL-5 and IFN-γ was determined by using the ELISPOT technique as previously described[16], with the exception that GAD65 and β-galactosidase (100 μg/ml) were used as antigens, and 11B11 together with biotinylated BVD6-24G2 (PharMingen, San Diego, California) was used for capture and detection of IL-4. The resulting spots were counted manually.

Adoptive transfer of diabetes. Eight-week-old NOD mice were injected i.p. with 100 μg GAD65 or control β-galactosidase in 100 μl of 50% IFA and reinjected at 14 weeks of age. Five weeks later, single-cell suspensions of splenic mononuclear cells were prepared from each group, as well as from unmanipulated diabetic NOD mice. Ten million splenic mononuclear cells from the unmanipulated diabetic mice were mixed with an equal number of splenic mononuclear cells from GAD65 or β-galactosidase-treated mice and injected intravenously into 5-week-old female NOD mice that had received 500 rad γ-irradiation[27]. Another control group received 1×10^7 splenic mononuclear cells obtained only from unmanipulated, diabetic mice.

T-cell proliferation assays. Female NOD mice were injected i.p. at 8 weeks of age with 100 μg GAD65, or control β-galactosidase, in 100 μl of 50% IFA. The mice were reinjected 2 weeks later. At

12 weeks of age, splenic T cells were tested for proliferative responses to GAD65, hsp65, β-galactosidase and the HEL peptide, as previously described[4].

IDDM incidence. At 8 weeks of age, groups of 10 female NOD mice were injected i.p. with 50 μg GAD65 or control β-galactosidase in 100 μl of 50% IFA. Another control group received 100 μl of 50% IFA alone. Because there may be a requirement for continual antigen presentation[28], the mice were reinjected every 6 weeks until 40 weeks of age. Urine glucose levels were monitored weekly for diabetes by Tes-tape (Lilly, Indianapolis, Indiana). After we observed abnormal glucose in the urine, blood glucose levels were monitored twice weekly. A recording of two consecutive blood glucose levels of >13 mmol/l was considered as IDDM onset.

Transplantation of islets. Female NOD mice were monitored for the onset of IDDM, after which the mice were maintained on 1.0–1.5 units insulin (Humulin U, Lilly) per day. At the time of IDDM onset, mice were injected with either 100 μg of GAD65, hsp65, insulin B-chain or control β-galactosidase i.p. in 50% IFA. Ten days later the mice were reinjected. Ten days after the second treatment, 3000 freshly isolated islets from newborn NOD mice were transplanted into the space beneath the kidney capsule, and humulin administration was discontinued. The mice were reinjected every 2 weeks. Recurrence of diabetes is defined as two consecutive blood glucose levels of >13 mmol/l.

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Neonatal Tolerization With Glutamic Acid Decarboxylase But Not With Bovine Serum Albumin Delays the Onset of Diabetes in NOD Mice

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To test the role of glutamic acid decarboxylase (GAD_{65}) or bovine serum albumin (BSA) autoimmunity in the pathogenesis of diabetes, GAD_{65} or BSA was injected intraperitoneally into neonatal female NOD mice (100 μg/mouse of each protein). Treatment with GAD_{65}, but not with BSA, significantly delayed the onset of diabetes compared with control mice ($P < 0.05$). At 18 weeks, 6 of 10 control mice compared with 0 of 10 GAD_{65}-treated mice ($P = 0.005$) and 7 of 14 BSA-treated mice had developed diabetes. However, after 79 weeks, 6 of 10 of the GAD_{65}-treated mice were diabetic compared with 9 of 10 of the control mice and 12 of 14 of the BSA-treated mice. In GAD_{65}-treated mice without diabetes, insulitis was markedly reduced compared with control or BSA-treated mice ($P < 10^{-4}$). To further elucidate why GAD becomes an autoantigen, the expression in NOD mice islets was studied. Quantitative immunohistochemistry revealed that islet cell expression of GAD was increased in 5-week-old NOD mice compared with BALB/c mice ($P = 0.02$). With the occurrence of insulitis (9–15 weeks), the GAD expression was further increased relative to 5-week-old NOD mice ($P < 0.02$). In conclusion, GAD, but not BSA, autoimmunity is important for the development of diabetes in NOD mice. Furthermore, concordant with the appearance of insulitis, the GAD expression increased in NOD mouse islets, which could possibly potentiate the β-cell–directed autoimmunity. *Diabetes* 43: 1478–1484, 1994

The onset of insulin-dependent diabetes mellitus (IDDM) is associated with several autoimmune phenomena, including mononuclear cell infiltration of the islets of Langerhans and the presence of circulating islet cell autoantibodies (1,2). One hypothesis to explain the highly selective mechanism of β-cell destruction includes the existence of β-cell autoantigens, which, for unknown reasons, may direct an autoimmune response toward the islets.

In this context, several antigens have been implicated. Recently, interest has been focused on bovine serum albumin (BSA) (3,4) and glutamic acid decarboxylase (GAD_{65}) (5–8). Antibodies as well as T-cell reactivity against GAD_{65} can be detected in the majority of patients with recent-onset IDDM (6,9–12) but also several years before clinical onset of the disease (13–15), suggesting that this antigen could be important in the early stage of the disease. Likewise, antibodies and T-cell reactivity against BSA are found in the majority of patients with recent-onset IDDM (3,4). Polyclonal antisera to BSA have been demonstrated to cross-react with an islet cell membrane protein referred to as P69, suggesting that a BSA immune response can trigger islet autoimmunity via mimicking epitopes (3,16).

Autoantibodies and T-cell reactivity to GAD_{65} as well as antibodies to BSA have also been reported in the NOD mouse (7,8,17). Immunological tolerance to recombinant GAD_{65} was recently shown to prevent or delay the onset of diabetes in NOD mice followed for 25–37 weeks (7,8), emphasizing the importance of GAD_{65} as an islet cell autoantigen in the pathogenesis of IDDM in NOD mice. However, these results are difficult to reconcile with the very low expression of GAD in mouse islets.

In this study, we have therefore addressed two questions. First, we have characterized the role of autoimmunity to BSA and GAD_{65} in NOD mice. The long-term effects of tolerization to either BSA or GAD_{65} affinity purified from rat brains were compared by following NOD mice for 79 weeks after neonatal tolerization. Second, we have studied the level of age-related islet GAD expression in NOD mice compared with other nondiabetic strains of mice.

RESEARCH DESIGN AND METHODS

Antibodies and animals. GAD_{67} rabbit antisera 1266 and 1263 were raised and characterized as previously described against the COOH-terminal and NH_2-terminal parts of rat GAD_{67}, respectively (18,19). GAD_6 monoclonal antibody, specific for GAD_{65} (19), was obtained from the Developmental Studies Hybridoma Bank (Baltimore, MD). The following mouse (all from Bomholtgaard Breeding and Research Centre, Ry, Denmark) and rat (all from Møllegaard Breeding Centre, Skensved, Denmark) strains were used for immunoblotting analysis: CD1 mice, BALB/c mice, NOD mice, Wistar rats, and Lewis rats. NOD mice used for tolerization were all female, and the GAD and BSA injections were done 24 h after birth. To induce tolerance, the antigens were diluted in phosphate-buffered saline (PBS) as previously described (20). Blood glucose measurements were determined with a Cobas Micra plus according to the manufacturer's instructions (Roche, Grenzach-Wyhlen, Germany). Mice with blood glucose levels >13 mmol/l for >6 days were considered diabetic. The tolerized NOD mice were maintained under specific pathogen-free conditions.

Purification of GAD_{65} from rat brains. Brains from male and female Wistar rats (12–25 weeks old) were removed, swollen on ice for 10 min

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IDDM, insulin-dependent diabetes mellitus; GAD, glutamic acid decarboxylase; BSA, bovine serum albumin; PBS, phosphate-buffered saline; HLB, hypotonic lysis buffer; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; ELISA, enzyme-linked immunosorbent assay; HSA, human serum albumin.

in 10 mmol/l HEPES (pH 7.4), 1 mmol/l MgCl, and 1 mmol/l EGTA (hypotonic lysis buffer [HLB]), and then homogenized with a Polytron (Kinematica, Lucerne, Switzerland). The homogenates were centrifuged at 100,000 *g* for 1 h at 4°C to obtain cytosolic and particulate fractions. The GAD_{65} protein was purified from the cytosolic fraction by immunoaffinity chromatography using the GAD_6 monoclonal antibody purified from culture supernatant and coupled to CNBr-activated Sepharose-4B, according to the manufacturer's instructions (Pharmacia, Uppsala, Sweden), at 2.5 mg IgG/ml gel. Using 1 ml of coupled gel in 10 × 50 mm columns (Pharmacia), the rat brain cytosolic fraction was applied at 0.25 ml/min at 4°C. The gel was washed in 10 vol of HLB containing 0.5 mol/l NaCl and then 10 vol of HLB. GAD_{65} was eluted with 100 mmol/l glycine (pH 3) and collected in tubes containing 100 µl of 1 mol/l phosphate buffer (pH 8.8). All eluted samples were analyzed for enzymatic activity and by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by Coomassie staining. Fractions containing the GAD_{65} protein were pooled, dialyzed against PBS at 4°C, and the protein concentration determined (Pierce, Rockford, IL). The affinity-purified GAD_{65} protein was analyzed by SDS-PAGE followed by Coomassie staining and immunoblotting analysis with GAD-specific antiserum (R1266), which showed the purity to be >95% (data not shown). Almost all of the contaminating proteins distinct from full-length GAD_{65} and small amounts of GAD_{67} were shown by immunoblotting analysis to be degradation products from GAD, α- and β-tubulin, and immunoglobulin leaking from the affinity column (data not shown). To our knowledge, none of these proteins are involved in the pathogenesis of diabetes and they consist of <1%, i.e., <1 µg/injected animal; therefore, they have not been removed.

GAD_{65} antibody assay. GAD_{65} autoantibodies were measured as previously described (12). Briefly, the human GAD_{65} cDNA (21) was transcribed and translated in vitro, according to the manufacturer's instructions (Promega, Madison, WI), in the presence of [^{35}S]methionine (Amersham, Amersham, U.K.). The in vitro–translated GAD_{65} was applied to a NAP 5 column (Pharmacia), and aliquots containing ~30,000 cpm of in vitro–translated GAD_{65} were used for immunoprecipitation with mouse serum with or without the addition of unlabeled affinity-purified recombinant human GAD_{65} as a competitor. The immune complexes were isolated with protein A-Sepharose (Pharmacia) and washed, and the amount of immunoprecipitated GAD_{65} was quantified by scintillation counting. All samples were tested in duplicate. GAD_{65} antibody levels were expressed as index values:

GAD_{65} index = [(cpm of sample − cpm of sample in competition with unlabeled GAD_{65})/ (cpm of positive control − cpm of positive control in competition with unlabeled GAD_{65})].

Sera were regarded as positive when GAD_{65} antibody index values exceeded the mean plus 2 SDs of GAD_{65} antibody indexes in 10 BALB/c, 10 NMRI, and 10 C57/Bl mice.

Enzyme-linked immunosorbent assay (ELISA) for detection of BSA antibodies. The BSA antibodies were detected as described previously (17). In brief, 96-microwell plates were coated overnight with 5 µg/ml BSA in 50 mmol/l Na_2CO_3/$NaHCO_3$ buffer (pH 9.6) and washed in PBS. Additional binding sites were blocked by incubation for 2 h at 37°C with gelatin in PBS. Mouse sera were diluted in PBS and added to the plates, which were incubated for 2 h at room temperature followed by washing in PBS and incubation for 1.5 h at room temperature with rabbit-anti-mouse IgG (H + L) peroxidase-conjugated antibodies (Zymed, San Francisco, CA). After washing, substrate was added (*O*-phenyldiamine and 0.03% hydrogen peroxide in water) for 30 min. The reaction was stopped by adding 2 mol/l H_2SO_4, and the absorbance was measured at 490 mm. In competition experiments, the ELISA was performed as described above, but free BSA or human serum albumin (HSA) (HSA was added to the wells at the same time as the mouse serum).

Immunohistochemistry. Mouse pancreases were fixed in 4% paraformaldehyde, 0.1 mol/l phosphate buffer (pH 7.2) for 24 h. The tissue was embedded in paraffin, and 3-µm sections were cut on a microtome. Immunostaining was done using a Histostain-SP streptavidin immunostaining kit (Zymed) for rabbit primary antibodies according to the manufacturer's instructions. The bound antibodies were detected by alkaline phosphatase conjugated to biotin (Dakopats, Glostrup, Denmark) according to the manufacturer's instructions. Stainings were examined on an Olympus microscope (Olympus, New Hyde Park, NY) and quantified by National Institutes of Health image analysis software.

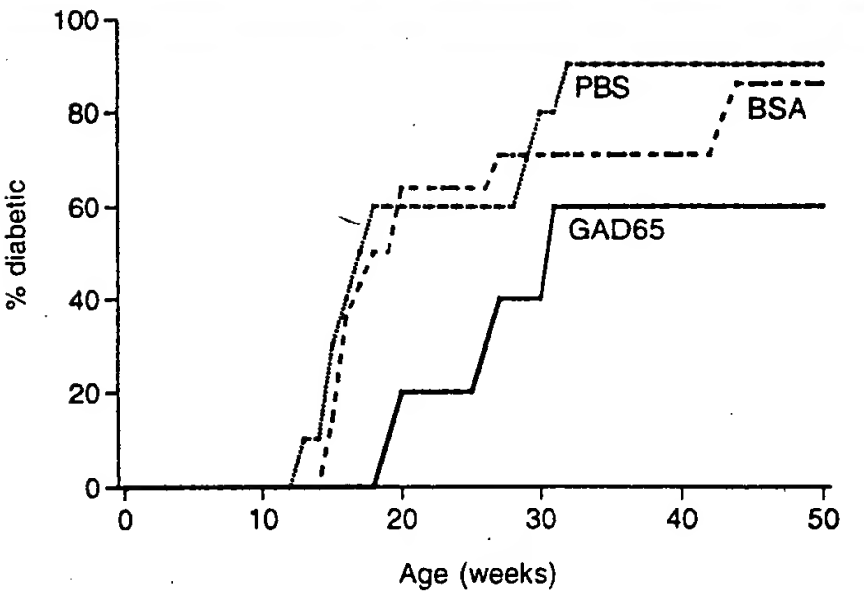

FIG. 1. Cumulative incidence of diabetes in female NOD mice tolerized intraperitoneally with BSA (n = 14), GAD_{65} (n = 10), or PBS (n = 10) alone. Mice with blood glucose levels >13 mmol/l for >6 days were considered diabetic. Mice were followed for 79 weeks (data not shown), but the diabetes incidence did not change after 50 weeks of age. GAD-treated mice showed a significant delay in diabetes onset compared with the BSA- and PBS-treated animals (P < 0.05).

Paraffin-embedded sections were analyzed for insulitis by staining with eosin and hematoxylin.

Western blotting. Islets, isolated as previously described (16), or brain tissue were swollen on ice for 10 min in HLB and then homogenized. The homogenates were centrifuged at 100,000 *g* for 1 h at 4°C to obtain a cytosolic and a particulate fraction. The cytosolic fraction was diluted in 20 mmol/l Tris buffer (pH 7.4) and 150 mmol/l NaCl, and the proteins were denatured under reducing conditions, subjected to 10% PAGE, and electroblotted onto nitrocellulose filters (22). Indirect immunostaining was done using [^{35}S]methionine-labeled protein A (Pharmacia). Staining intensities were quantified on a Phosphor Image analyzer (Molecular Dynamics, Kemsing, U.K.).

Statistical analysis. Statistical analyses included Fisher's exact test, Student's t test, and Mann-Whitney U test. The level of significance was chosen as 5%.

RESULTS

Neonatal tolerization to GAD_{65} and BSA: effects on the development of insulitis and diabetes. Female NOD mice were injected once intraperitoneally 24 h after birth with either 100 µg BSA (n = 14) or GAD_{65} (n = 10) in PBS. Control mice received only PBS (n = 10). The median age at onset of diabetes was similar in the BSA- and PBS-injected animals (17 weeks, range 15–44 weeks; 17 weeks, range 13–32 weeks, respectively) and similar to the expected age of spontaneous onset in the parent colony (median 17 weeks, range 11–29 weeks). In contrast, the median age at onset of diabetes in the GAD-injected animals was significantly higher (27 weeks, range 19–31 weeks) than in both the BSA- and PBS-injected animals (P < 0.05) (Fig. 1). This delay was most pronounced at 18 weeks, i.e., 6 of 10 control mice compared with 0 of 10 GAD_{65}-treated mice (P = 0.005) and 7 of 14 BSA-treated mice had developed diabetes. However, after 79 weeks, 6 of 10 of the GAD_{65}-treated mice were diabetic compared with 9 of 10 of the control mice and 12 of 14 of the BSA-treated mice.

To further ascertain the effect of tolerization with GAD_{65} and BSA, the pancreases from diabetic and nondiabetic NOD mice were analyzed for the degree of mononuclear cell infiltration in the islets (insulitis). There were no significant differences in the degree of insulitis among the diabetic NOD mice from any groups (Fig. 2). However, histological examination of the pancreases from nondiabetic GAD_{65}-injected

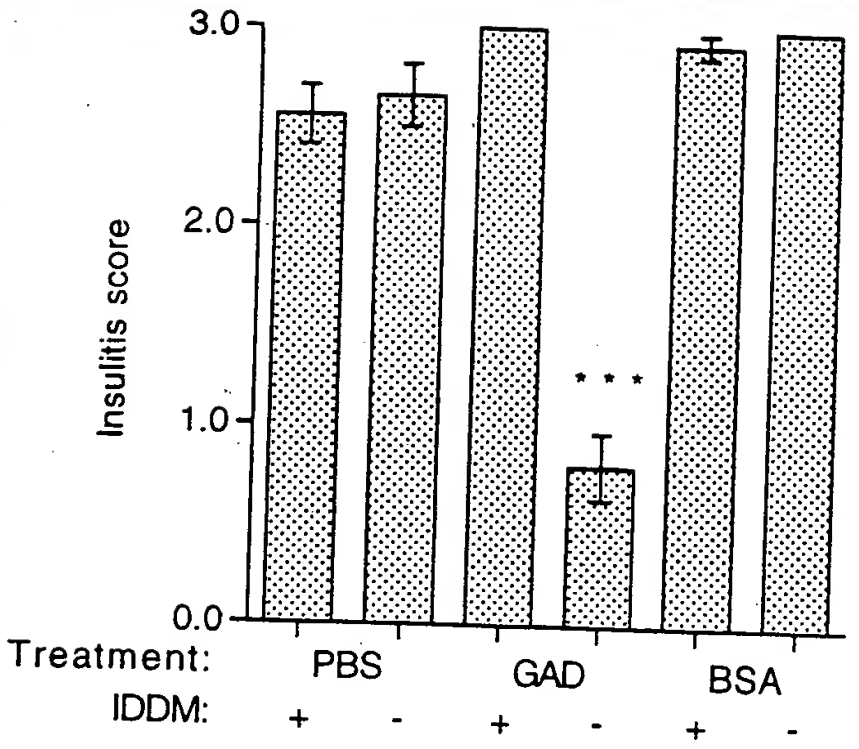

FIG. 2. Characterization of insulitis in diabetic (+) and nondiabetic (−) female NOD mice tolerized intraperitoneally with BSA (n = 14), GAD_{65} (n = 10), or PBS (n = 10) alone. The severity of insulitis was assessed by the following criteria: 0, islets free of insulitis; 1, <33% of the islet infiltrated; 2, between 33 and 50% of the islet infiltrated; and 3, >50% of the islet infiltrated. Approximately 10 islets from each animal were analyzed. ***$P < 10^{-4}$, nondiabetic GAD-treated NOD mice (4 of 4) vs. BSA- and PBS-treated diabetic (4 of 12 and 4 of 9, respectively) and nondiabetic (2 of 2 and 1 of 1, respectively) mice.

mice (n = 4) revealed a significant reduction in the intra-islet cell infiltration ($P < 10^{-4}$) compared with both the diabetic mice and the nondiabetic BSA-treated (n = 2) or PBS-treated (n = 1) NOD mice (Fig. 3).

Effects on antibody formation. Prospectively sampled sera were analyzed for the presence of antibodies to GAD_{65} and BSA. GAD_{65} antibodies were quantified by a radioligand assay using in vitro transcribed and translated recombinant human GAD_{65} as tracer. None of the 113 serum samples from NOD mice injected with either BSA (n = 14) or PBS (n = 10) contained GAD_{65} autoantibodies (Fig. 3*A* and *B*). Surprisingly, among the GAD_{65}-treated NOD mice, three of four of the nondiabetic and two of six of the diabetic animals were positive for GAD_{65} antibodies (Fig. 3*C*). In some animals, GAD_{65} antibodies were first detected in serum samples obtained 11–19 weeks after the injection, but not in samples obtained before, indicating that they were not the result of an immunization to the injected GAD_{65} (Fig. 3*C*).

BSA antibodies were detected using a quantitative ELISA technique. Only 14% (2 of 14) of the BSA-treated animals had BSA antibodies that were significantly lower than in the GAD_{65}- and PBS-treated mice (60% [6 of 10] in both groups [$P < 0.01$]) (Fig. 3*D*, *E*, and *F*), thus demonstrating that neonatal injection of BSA inhibited the normal occurrence of BSA immunity found in adult NOD mice. There was no correlation between the presence or level of BSA antibodies and the development of diabetes (Fig. 3*D*, *E*, and *F*). The binding of NOD sera to BSA could, in all cases, be displaced by incubation with an excess of BSA. However, since HSA could not inhibit binding (data not shown), the BSA epitopes recognized by NOD sera are not present in HSA.

Characterization of GAD expression in rat and mouse islets. Immunohistochemistry with a GAD_{65}/GAD_{67}-specific peptide antiserum (R1266) was used to quantify the GAD expression in NOD mouse pancreases at 5, 9, 12, and 15 weeks of age (n = 3 in each age-group). The expression of GAD increased from week 5 to 15 ($P < 0.02$). This increase was gradual and correlated with the occurrence of insulitis (Fig. 4). Comparing GAD expression in NOD and BALB/c mice showed that pancreases from 9-week-old BALB/c mice (n = 3) contained less GAD than age-matched NOD mice (n = 3) ($P < 10^{-4}$) (Fig. 4).

Since the antiserum (R1266) used for immunohistochemistry recognized both GAD_{65} and GAD_{67}, it was not possible by this technique to refer the level of GAD expression to the individual isoforms. We therefore quantified GAD_{65} and GAD_{67} expression by immunoblotting analysis. Islet and brain tissue from age-matched (7- to 9-week-old) CD1, BALB/c, and NOD mice as well as Wistar and Lewis rats were analyzed using the GAD antiserum R1267 reacting to mouse and rat GAD_{65} and GAD_{67}. This analysis confirmed that NOD mouse islets express more GAD than do the nondiabetic CD1 and BALB/c mice islets, i.e., the GAD_{65} expression was 25–38% lower and the GAD_{67} expression was 12–13% lower (Table 1). Quantifying the isoform-specific GAD expression in rat islets revealed that both rat strains expressed ~1.6- to 2-fold more GAD_{65} than GAD_{67} (Table 1). In contrast, mouse islets from the three different strains expressed about three- to fourfold more GAD_{67} than GAD_{65}, but the overall expression of GAD was much lower compared with rat islets. Thus, rat islets contained ~10- to 20-fold more GAD_{65} and 2- to 2.5-fold more GAD_{67} than did mouse islets (Table 1). These differences in levels of GAD expression between rat and mouse are islet-specific, because rat and mouse brain tissue showed similar high levels of GAD (Table 1).

DISCUSSION

We show here that neonatal injection with GAD_{65} protects NOD mice from the development of diabetes, whereas neonatal treatment with BSA does not affect diabetes development compared with control-treated mice (Fig. 1). Nondiabetic mice from the GAD_{65}-injected group displayed a nearly complete absence of intra-islet mononuclear cell infiltration, in contrast to the BSA- and PBS-injected mice without IDDM (Fig. 2). This observation suggests that GAD_{65} injection prevented not only diabetes but also other islet cell autoimmune phenomena. This study also demonstrates the importance of a long follow-up period of NOD mice treated with GAD_{65} or other immunomodulatory agents to ascertain the full effects of the therapy. During the first 18 weeks, none of the GAD_{65}-treated NOD mice developed diabetes. In contrast, 60% (6 of 10) of the PBS-treated animals had already developed disease (Fig. 1). These effects were only temporary, however, since 60% (6 of 10) of the GAD_{65}-treated NOD mice developed diabetes during the 60-week observation period that followed (Fig. 1). Nevertheless, these findings argue in favor of a critical role of GAD_{65} autoimmunity in the pathogenesis of IDDM in NOD mice.

In humans, GAD_{65} autoantibodies and T-cell reactivity are found in 70–90% of recent onset IDDM patients (6,9–12). The occurrence of GAD_{65} autoimmunity several years before the clinical onset of disease (13–15) suggests a primary role of this autoantigen in the early stage of disease. However, whether the autoimmune response to GAD_{65} is the consequence or the cause of β-cell destruction is not known at present.

In several neurological diseases, neurons die and, consequently, their content is exposed to the immune system

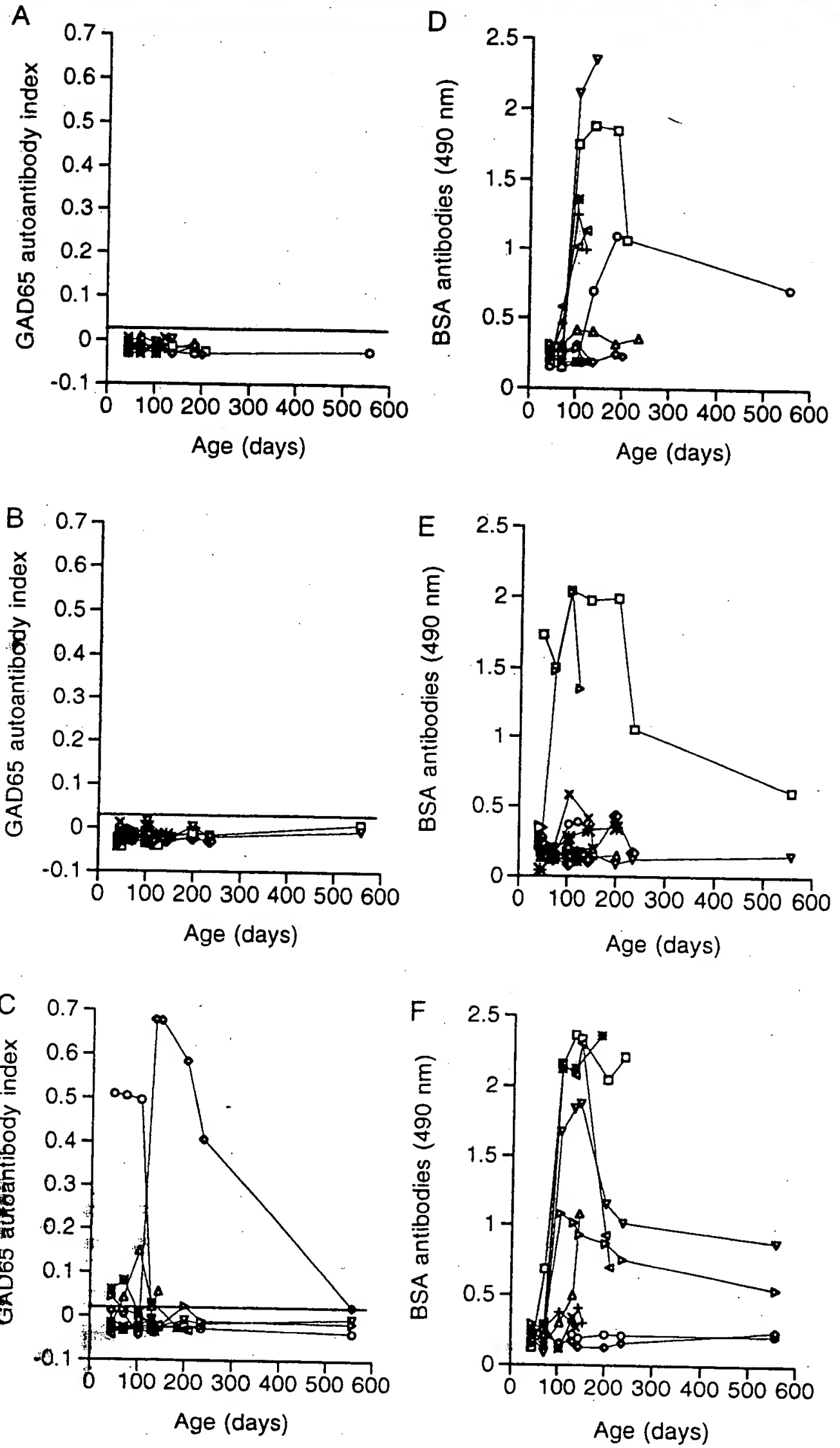

FIG. 3. Characterization of GAD_{65} (*A*, *B*, and *C*) and BSA (*D*, *E*, and *F*) antibodies in sera sampled prospectively in female NOD mice tolerized intraperitoneally with PBS ($n = 10$) alone (*A* and *D*), BSA ($n = 14$) (*B* and *E*), or GAD_{65} ($n = 10$) (*C* and *F*). The prevalence of GAD_{65} autoantibodies was significantly higher in the GAD_{65}-treated animals (50%; 5 of 10) vs. the BSA- and PBS-treated animals (0%; 0 of 14 and 0 of 10, respectively), $P < 0.001$. The prevalence of BSA antibodies was significantly lower in the BSA-treated animals (14%; 2 of 14) vs. the GAD_{65}- and PBS-treated animals (60%; 6 of 10 in both groups), $P < 0.01$.

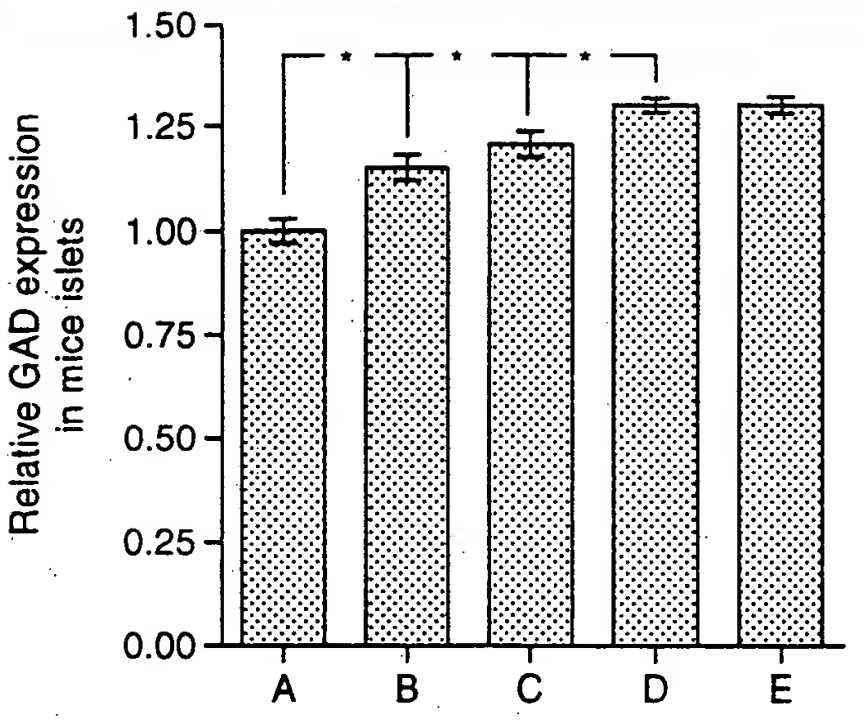

FIG. 4. Immunohistochemical analysis of GAD_{65} and GAD_{67} expression in formalin-fixed paraffin-embedded pancreases removed at 9 weeks from BALB/c mice (*A*) and at 5 (*B*), 9 (*C*), 12 (*D*), and 15 (*E*) weeks of age from NOD mice (n = 3 in each age-group). The sections were stained with an antiserum recognizing both mouse GAD_{65} and GAD_{67} (R1266) and quantified as described in METHODS (characterization of the R1266 antiserum is described in the legend to Table 1). *$P < 0.02$, 9-week-old BALB/c mice vs. 5- to 15-week-old NOD mice, 5-week-old NOD mice vs. 9-week-old NOD mice, and 9-week-old NOD mice vs. 12-week-old NOD mice.

without initiating an autoimmune response to GAD. It would therefore seem unlikely that the mere release of GAD would cause autoimmunity as a result of islet cell destruction in IDDM. The data rather suggest that the prediabetic immune system specifically is being stimulated toward GAD_{65} reactivity and that GAD autoimmunity may play a direct role in the disease pathogenesis. Thus, tolerizing NOD mice with recombinant GAD_{65} from different species and sources, i.e., human (7) and mouse (8), expressed in *Escherichia coli* or Sf9 insect cells, respectively, as well as with native affinity-purified GAD_{65} from rat brains used in this study, have been shown to prevent or delay the onset of diabetes.

These observations indicate a primary role of GAD_{65} autoimmunity in the development of disease. However, untreated NOD mice without diabetes at 24 weeks of age have also been reported to have high T-cell proliferative responses to GAD_{65} (8), even though they are not very likely to develop the disease at a later time (8). Although T-cell reactivity to GAD_{65} and GAD_{67} is detectable in NOD mice already at 4 weeks, the response to whole islet extract is even stronger at this early stage (8). Since we have demonstrated that mouse islets contain only very little GAD, the reported T-cell reactivity against mouse islet proteins will most likely have to be explained by other autoantigens. Taken together, these observations show that although GAD autoimmunity is necessary, it is not sufficient for the development of NOD mouse diabetes. This is further supported by the fact that mice immunized with high doses of recombinant GAD_{65} (n = 20 from four different strains of mice, now immunized three times with GAD and followed for 8 weeks) all have normal glucose levels (A. Plesner, A.W., T.D., J.S.P., unpublished observations). Thus, although GAD_{65} tolerization can prevent the development of spontaneous diabetes in NOD mice, disease is not easily induced by immunization.

Autoantibodies to GAD_{65} have been reported in NOD mice (8), but because we and others (23,24) have not been able to detect GAD_{65} antibodies in untreated mice, their levels may be very low or absent. It was therefore surprising that 5 of 10 of the GAD_{65} NOD mice were positive for GAD_{65} antibodies, since the neonatal tolerization could be expected to inhibit the immune response (Fig. 3*B*). The GAD_{65} antibodies appeared in some animals as late as 11–19 weeks after the injection and were therefore not likely to result from immunization to the injected GAD_{65} (Fig. 3*C*). $CD4^+$ Th_1- and Th_2-cells are characterized by promoting cellular or humoral immunity, respectively (25). Furthermore, $CD4^+$ Th_2-cells have been suggested to inhibit $CD4^+$ Th_1-dependent cellular immunity by secretion of interleukin-4 (among others), and $CD4^+$ Th_1-cells have been suggested to inhibit $CD4^+$ Th_2-dependent humoral immunity by secretion of γ-interferon (24). The apparent paradox that untreated NOD mice have no detectable GAD autoantibodies and that GAD_{65}-injected mice do have them could therefore be explained if neonatal GAD_{65} treatment promotes/induces $CD4^+$ Th_2-cells, which will stimulate humoral immunity and inhibited $CD4^+$ Th_1-dependent cellular immunity. GAD_{65}-specific $CD4^+$ T-cells from untreated NOD mice have been shown to secrete high amounts of γ-interferon (7). The lack of GAD_{65} antibodies in BSA-treated, PBS-treated, and untreated NOD mice could therefore be explained by a predominant Th_1 response in these diabetes-prone animals. In concordance with these speculations, it has been shown that administration of interleukin-4 to NOD mice completely prevented diabetes, although this study provided no data regarding induction of Th_2- versus Th_1-cells (26).

The autoantigenic properties of GAD in NOD mice islets are not well understood, but the level of GAD in the islet would be expected to influence the autoimmune process. We therefore investigated islet GAD expression in NOD mice compared with other nondiabetic strains of mice. Intriguingly, NOD mouse islet GAD levels were high when com-

TABLE 1
GAD_{65} and GAD_{67} levels in different rat and mouse strains analyzed by quantitative immunoblotting analysis

Strain	Islet GAD_{65}	Islet GAD_{67}	Brain GAD_{65}	Brain GAD_{67}
NOD	1.00 ± 0.00	3.14 ± 0.23	10.29 ± 0.05	11.41 ± 2.16
BALB/c	0.75 ± 0.01	2.78 ± 0.16	12.33 ± 1.74	10.80 ± 1.67
CD1	0.62 ± 0.06	2.73 ± 0.01	10.67 ± 0.67	9.88 ± 0.21
Wistar	11.58 ± 1.94	6.95 ± 0.55	21.71 ± 1.35	12.78 ± 2.09
Lewis	13.06 ± 3.68	6.65 ± 0.36	22.99 ± 1.56	11.67 ± 1.35

Data are means ± SD. Protein (10 μg/lane) from either islets or brain was used. The values are expressed relative to NOD islet GAD_{65}. Characteristics of epitopes recognized by the antiserum (R1266) used to quantify the GAD isoform expression were as follows. The R1266 antiserum was raised to a 19–amino acid peptide at the COOH-terminal end of rat GAD_{67}, which differs from rat GAD_{65} by two amino acids. However, comparing immunoprecipitation analysis of [^{35}S]methionine-labeled rats islets using GAD-specific antibodies (GAD_6 and R1263), removing all GAD present in the islet extract, with immunoblotting of the same amounts of islets using the R1266 antiserum, demonstrated that the R1266 antiserum reacts equally well with GAD_{65} and GAD_{67} (data not shown). Since the amino acid sequence in the peptide used for immunization is identical in rat and mouse GAD_{65} (24), similar reactivity of R1266 to rat and mouse GAD_{65} is expected, which has also been confirmed by others (24). Mouse GAD_{67} differs at three neutral amino acids within the 19–amino acid rat GAD_{67} peptide used for immunization (24). However, the finding of similar levels of expression of GAD_{65} and GAD_{67} in rat and mouse brain suggest that the amino acid difference does not affect the antibody binding.

pared with nondiabetic strains of mice (Fig. 4 and Table 1). This elevated GAD expression seemed to correlate with the occurrence of insulitis, i.e., the GAD expression gradually increased from 5 to 15 weeks of age ($P < 0.02$). Whether this increase is caused by lymphokines released during the insulitis process or by the increased metabolic demand on the remaining β-cells is not known. Findings from several in vitro studies have demonstrated that high glucose increases the GAD_{65} expression (27,28). The NOD mice pancreases analyzed between 5 and 12 weeks of age, which all showed elevated GAD expression compared with BALB/c mice (Fig. 4 and Table 1), were, however, normoglycemic (data not shown). These data suggest that high glucose levels by themselves are not responsible for the increased GAD expression observed in the in vitro studies, but rather that the effect of glucose on GAD expression is mediated by the increased functional demand on the pancreatic β-cells associated with the decline of β-cell mass in the prediabetic NOD mice. Several studies have demonstrated that prophylactic insulin treatment in NOD mice decreases the incidence of diabetes (29,30). It is tempting to believe that these data could be explained by an inhibition of the functional activity of the β-cells and their subsequent decreased GAD expression. Taken together, these data suggest that the increased GAD expression in NOD mice islets could potentiate the autoimmune response, leading to disease.

Cow's milk proteins have been implicated as a possible trigger of β-cell autoimmunity and IDDM (3). Studies in NOD mice and humans have revealed that almost all diabetic patients and NOD mice have antibodies to BSA (3,17). Additionally, antibodies raised against a 17–amino acid BSA peptide, known as the ABBOS peptide, were found to cross-react with a β-cell membrane protein (P69), which may represent the target antigen for milk-induced β-cell–specific immunity (3). Our study confirmed the presence of BSA antibodies in NOD mouse sera. The fact that the binding could not be inhibited with HSA (data not shown) indicates that the epitope recognized could be ABBOS, since the ABBOS peptide is different in HSA. However, we have now shown that tolerization with BSA significantly reduced the prevalence of BSA antibodies, but did not affect the occurrence of diabetes (Fig. 3), and there was no correlation between onset of disease and the level of BSA antibodies. It is therefore unlikely that BSA immunity is of importance in the pathogenic process leading to diabetes in NOD mice.

In conclusion, the findings presented here strongly indicate that autoimmunity toward GAD_{65}, in contrast to BSA, is important in the pathogenesis of NOD mouse diabetes. Furthermore, the increased GAD expression observed with the occurrence of insulitis could be speculated to amplify or even cause the autoimmune response leading to disease.

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IMMUNOLOGY
A Short Course

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changing the physical state of such tolerogenic substances by heating, which causes aggregation of proteins, or by incorporation into adjuvants converts them to potent immunogens.

4. ***Dosage*** of the substance may be critical. An important observation, first made by Mitchison, is that ***tolerance can be induced by opposite extremes of dosage***. The initial studies, showing that tolerance can be induced in adult animals, were accomplished by the use of relatively large doses of antigen, given repeatedly over long periods of time. Very small doses given similarly were also found to induce tolerance. Intermediate doses resulted in immunity.

5. ***Non-metabolizable substances*** may be tolerogenic. Pneumococcal polysaccharides and synthetic D-polypeptides (made with D rather than L isomers of amino acids) are resistant to enzymatic digestion. These substances are immunogenic when administered in very low quantities (1 μg), but slightly higher doses (10 μg) induce a long-lasting state of specific tolerance. One contributing factor in this case is that, although pneumococcal polysaccharide SIII (for example) is phagocytized, it is not digested and is repeatedly released back into circulation, where it can bind to antibody and prevent its detection in serum.

ROLE OF ACCESSORY CELLS IN TOLERANCE

The following observations led to the conclusion that an ***antigen-processing cell,*** like the macrophages discussed previously, is central to the outcome of the immune response. Generally, if antigen reaches this type of cell, immunity results; ***if the processing cell is bypassed, some form of tolerance is induced***. Thus, destruction of accessory cells by various agents, before antigen is given, leads to tolerance, and tolerance is easily induced in newborns with small or absent populations of accessory cells. Furthermore, differences in the ease with which tolerance can be induced in certain mouse strains reside in the properties of macrophage-like cells.

ROLES OF T AND B LYMPHOCYTES IN TOLERANCE

Soon after the phenomenon of cooperation between T and B cells in humoral immune responses became established, the conceptual and experimental approach to the study of tolerance was reappraised. The immediate questions dealt with the issue of whether the T or the B cells were affected by

DIAMYD
MEDICAL

Press Release

For immediate release
Minneapolis, MN * January 17, 2000

DIABETES VACCINE COMPLETES PHASE-I CLINICAL TRIALS.

Diamyd Inc., a biotech company with headquarters in Stockholm, Sweden, announces that the bulk product for its type-1 diabetes vaccine, Diamyd™, has completed a Phase-I clinical study in man.

This was reported by **Dr. John Robertson**, Director of Research & Development, at a scientific meeting in Miami organized by the National Institute of Diabetes & Digestive & Kidney Diseases on January 10th.

"The successful completion of our Phase-I clinical study, basically gives green light for further development of the Diamyd™ diabetes vaccine" concluded Dr. Robertson. As a consequence, the company plans to seek permission to conduct a Phase-II clinical study in about three months.

Diamyd™ is the proprietary name for recombinant human GAD65 (glutamic acid decarboxylase). The company´s development is based on its strong Intellectual Proprietary Rights for the GAD-molecule including several exclusive licensing agreements with the University of Gainesville, FL, the University of Washington, WA, and the UCLA, CA.
"The Diamyd vaccine is planned to reach the market by the end of year 2004", says **Anders Essen-Moller**, president and CEO of Diamyd Medical.
"If successful, we are looking at US$ 360 million in sales by year 2005 which according to our calculations corresponds to 2% of the potential market and a price of US$ 1500 per vaccination. We hope to strike an agreement with a suitable partner in due time and if so the company estimates its net income to US$ 70 million for the year 2006 and to US$ 100 million for the year 2007".

As previously reported in the scientific literature, several studies with GAD65 using the mouse model for the disease have shown that:

1) Transfer of GAD reactive lymphocytes between animals transfers disease.

2) Prevention of GAD-expression in beta-cells prevents disease

3) Administration of GAD to diabetes prone animals prevents disease

Based on these efficacy data, Diamyd Medical is currently developing GAD65 as a biopharmaceutical for treatment and/or prevention of type-1 diabetes. Results of preclinical studies have previously confirmed that there were no concerns regarding the use of the DiamydTM Bulk Product for Phase I.

The Phase I trial was designed solely to address clinical safety of the product and was started in February 1999. 24 healthy male Caucasian volunteers were selected for inclusion in this double-blind study involving sub-cutaneous injection of one of 4 ascending dose levels up to a maximum of 0.5mg/person. Additional inclusion criteria for this study were the absence of HLA DR3/DQ2 and HLA DR4/DQ8 genes, and absence of autoantibodies to GAD65, insulin, or IA-2.

The study outcome was as follows:

1. There were no adverse clinical effects

2. A dose of 0.5mg/person was well tolerated

3. Auto antibodies to GAD65, insulin or IA-2 were not induced

In April this year Diamyd Medical plans to apply for regulatory approval to conduct a multicenter Phase II study in LADA (Latent Autoimmune Diabetes in the Adult) patients in Europe. This study will involve subcutaneous administration of up to 0.5mg/person via a prime and boost regimen using GMP quality DiamydTM formulated with the humoural adjuvant alum. The Phase II study is intended to enable selection of doses and surrogate tolerance assays for further clinical development.

There are about 1.5 million diagnosed type-1 diabetes patients in the US alone according to the National Diabetes Foundation. In addition recent evidence suggests that an equal amount of patients originally diagnosed as type-2 diabetes patients in fact have the type-1 form of the disease.

A successful Diamyd vaccine could:

1 protect individuals at increased-risk for type-1 diabetes

2 preserve residual beta cell mass in recent-onset type-1 diabetes patients

3 prevent type-1 disease in patients diagnosed with the type-2 form of the disease

4 prevent recurrence of type-1 diabetes in patients receiving pancreas or islet cell transplantation.

The text in this press release contains certain historical and forward looking statements. Such statements include a number of risks and uncertainties and no guarantees whatsoever are given that such statements do not contain errors or that any plans or predictions will be realized. A more complete description of risks is available at Diamyd Medical and should be read before making an investment in

the company?s stock. A description of the company is available in the Annual Report dated November 1999.

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DIAMYD MEDICAL

MAIN MENU

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Press Release

Stockholm, June 14, 2003

DIAMYD REPORTS SUCCESSFUL CLINICAL PHASE II TRIAL WITH DIABETES VACCINE

Diamyd Medical AB, publicly traded on the Stockholm Stock Exchange in Sweden (O-list), today reported a positive outcome from a phase II trial with its GAD-based diabetes vaccine Diamyd™. The results may lead to a new treatment to prevent type 1 diabetes.

The presentation of the results took place at the American Diabetes Association (ADA) convention in New Orleans, by Dr. Åke Lernmark, University of Washington, Seattle. "My opinion is that this phase II study has been tremendously successful", says Åke Lernmark "Not only is it now shown that the Diamyd™ vaccine can be safely administrated in a wide range of doses, but a clear and significant positive effect (P=0.01) of the vaccine was found at one of the dose levels six months from first vaccination. It is also important to note that the trial was conducted to the highest standards which ads further weight to its results."

"We could not have hoped for better results. The Diamyd™ vaccine is safe and we have an effective dose to go on with", says Anders Essen-Möller, President and CEO of Diamyd Medical.

In type 1 diabetes, the immune system mistakenly destroys the insulin-producing cells in the pancreas in an autoimmune attack. Over time, this attack leads to a lack of insulin, the hormone that controls blood sugar levels. People with type 1 diabetes must inject insulin daily.

In type 2 diabetes, patients normally continue to produce their own insulin but are less sensitive to it. Therefore these patients may be treated with tablets to increase their sensitivity to insulin. A large group (about 10%) of the type 2 diabetes patients have antibodies to GAD. These patients are called LADA and suffer from a similar autoimmune attack as the type 1 diabetes patients, which leads to the need for insulin injections.

Diamyd Medical conducted the phase II clinical trial by vaccinating patients with recently diagnosed LADA. The GAD-vaccine successfully improved these patients' C-peptide levels and therefore their ability to make insulin over a six-month period, compared with patients who received a placebo.

"The study shows that the vaccine is safe and that it is possible to inhibit the autoimmune attack on the cells that make insulin, thereby slowing the progression of the disease," said Essen-Moller.

The vaccine to prevent type 1 diabetes arose from experiments with diabetes prone-mice that were protected from developing the disease by injecting GAD-protein. "It's tremendously satisfying to see our work at UCLA go from the lab to a clinical application with the potential to help so many people" said Daniel Kaufman, Ph.D., Professor, UCLA Department of Molecular and Medical Pharmacology, whose research team was first to develop and test a GAD-vaccine in diabetes-prone mice.

Diamyd Medical's phase II trial was conducted on 47 diabetes patients with the GAD-based vaccine Diamyd™ at the UMAS hospital in Malmoe and St.Gorans Hospital in Stockholm, Sweden. The patients were randomly divided into four groups with 12 patients in each group. Each patient received one first injection of Diamyd™ followed by at least

one boost injection four weeks after. Nine patients in every group received active drug whereas three received placebo. The groups received different doses of the vaccine ranging from 4 to 500 micrograms per dose. All patients visited the hospitals 10 times during this six-month study, and detailed clinical, immunological as well as neurological investigations showed no safety concerns at the administered dose levels.

The study results show that the diabetes vaccine significantly improves the serum C-peptide levels both at fasting (P=0.01) and after meals (P=0.02) at one of the doses.

"Since the vaccine seem effective when given to people with an advanced disease, we are hopeful that it will be highly effective when given at earlier stages of the disease process—we now know that type 1 diabetes takes years to develop and that we can detect people who are at early stages of the disease process by testing for GAD autoantibodies in their blood" said Essen-Moller

"We will now continue to analyze the results from this study" says Essen-Möller. The future for the Diamyd™ diabetes vaccine is promising".

Diamyd Medical is identifying and developing therapeutic candidates through phase II. The Company's intention is thereafter to seek co-operation with established pharmaceutical companies for further development. Diamyd Medical is pursuing various GAD-based development projects of which the GAD-based diabetes vaccine Diamyd™ is the most advanced at this time. Diamyd Medical has licensed exclusive and worldwide intellectual rights for therapeutic use of GAD from the Universities of California in Los Angeles and University of Florida in Gainesville, Florida.

The first application for Diamyd™ is older patients with adult onset diabetes with GAD antibodies since this patient group progress to full insulin dependence within a few years. The market for this application may be in the area of one billion US dollars per year. Future studies will address whether the vaccine can also prevent the development of type 1 diabetes in young people that have not yet developed the disease. With the availability of a potential therapeutic, the pre-diagnostic tests for who is at risk for developing the disease (based on the detection of antibodies to GAD and other islet proteins), becomes quite valuable. Diamyd has an extensive array of pre-diagnostic kits for detecting autoantibodies to these proteins. Additional possible applications of the vaccine are to prevent recurrent autoimmune diabetes after transplantation of islet cells and stem cell therapy.

About Diamyd Medical:

Diamyd Medical's business idea is to identify and develop pharmaceutical projects up to and including Phase II. At present Diamyd Medical is running a number of GAD-based development projects and has the licensed rights for this from universities in the US.

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Identification of the 64K autoantigen in insulin-dependent diabetes as the GABA-synthesizing enzyme glutamic acid decarboxylase

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The pancreatic islet β-cell autoantigen of relative molecular mass 64,000 (64K), which is a major target of autoantibodies associated with the development of insulin-dependent diabetes mellitus (IDDM) has been identified as glutamic acid decarboxylase, the biosynthesizing enzyme of the inhibitory neurotransmitter GABA (γ-aminobutyric acid). Pancreatic β cells and a subpopulation of central nervous system neurons express high levels of this enzyme. Autoantibodies against glutamic acid decarboxylase with a higher titre and increased epitope recognition compared with those usually associated with IDDM are found in stiff-man syndrome, a rare neurological disorder characterized by a high coincidence with IDDM.

THE cell-specific destruction of pancreatic β cells, which precedes the clinical onset of insulin-dependent diabetes mellitus is believed to be mediated by autoimmune mechanisms[1]. The autoimmune phenomena associated with the disease include massive lymphocytic infiltration of islets[2] and circulating autoantibodies to β cells[3]. A 64K β-cell autoantigen is a target of autoantibodies in this disease[4]. The 64K autoantibodies are present in ≥80% of newly diagnosed IDDM patients and have been detected up to several years before clinical onset of IDDM concomitant with a gradual loss of β cells[5–7]. The 64K antigen was found to be β cell-specific in an analysis of several tissues which did not include the brain[8]. The 64K autoantigen in β cells is detected as a hydrophilic soluble 65K form and a 64K hydrophobic form which can be both membrane bound and soluble (H. Schierbeck, L. Aagaard and S.B., manuscript submitted). The 64K forms can be resolved into two components, α and β (ref. 9). The function of the 64K protein in the β cell has remained elusive.

Most patients with a rare but severe neurological disease called stiff-man syndrome (SMS) have autoantibodies to GABA-secreting neurons. Glutamic acid decarboxylase (GAD), the enzyme that synthesizes GABA from glutamic acid, is the predominant autoantigen[10,11]. Surprisingly, almost all patients positive for the autoantibody to GABA-secreting neurons are also positive for islet cell cytoplasmic antibodies, as demonstrated by immunofluorescence of pancreatic sections, and a significant fraction have IDDM[11]. GAD is selectively expressed in GABA-secreting neurons in the central nervous system (CNS)[12]. Outside neurons, GAD is found at high levels in pancreatic β cells[13–15]. There are at least two isomers of GAD in brain, which are resolved by differences in their mobility on SDS-polyacrylamide

gel electrophoresis; their molecular weights have been described as 59–66K (ref. 16).

Because IDDM is the autoimmune disease that is most often associated with SMS and because some of the characteristics of GAD and the 64K autoantigen are similar, we hypothesized that they were the same protein. Here we demonstrate that the 64K autoantigen in IDDM is the same as GAD in pancreatic β cells.

Immunoprecipitation of 64K autoantigen

We first assessed whether serum S3, a sheep antiserum raised against purified rat brain GAD[17], and sera from SMS patients positive for GAD antibodies[10,11], could immunoprecipitate the 64K autoantigen from rat islets. We used [^{35}S]methionine-labelled rat islet cell fractions partially enriched for the 64K antigen (S-100 DP, Fig. 1). The sera positive for GAD antibody (anti-GAD sera) immunoprecipitated a doublet of [^{35}S]methionine-labelled proteins, which on SDS-PAGE have the same mobility as the 64K α/β autoantigen immunoprecipitated by IDDM sera[7] (Fig. 1*a*, lanes 4–9).

To assess whether the proteins recognized by the anti-64K-sera and by anti-GAD sera were the same, supernatants resulting from immunoprecipitation with anti-GAD sera were subsequently reprecipitated with anti-64K IDDM sera. Similarly, supernatants resulting from immunoprecipitation with anti-64K IDDM sera were reprecipitated with anti-GAD sera. Results from those experiments showed that anti-GAD sera, and anti-64K sera each quantitatively removed the protein recognized by the other group of sera, demonstrating complete cross-reactivity between anti-GAD sera and anti-64K sera, and strongly suggesting that the 64K protein is the same as GAD in rat islets (Fig. 1*a*, lanes 10–20).

Trypsin digestion of [^{35}S]methionine-labelled islet cell extracts followed by immunoprecipitation showed that a 55K immunoreactive fragment was formed from both the 64K protein and GAD, further verifying their common identity (Fig. 1*b*). Furthermore, two-dimensional analyses of GAD immunoprecipitated from [^{35}S]methionine-labelled islets with the S3 antiserum revealed an identical pattern to that described for the 64K protein (H. Schierbeck, L. Aagaard and S.B., manuscript submitted; and ref. 9) (results not shown). This pattern was also the same as the two-dimensional pattern of pancreatic and brain GAD shown by western blotting of two-dimensional gels with the S3 serum (see Fig. 5*a*).

Immunoprecipitation of GAD

We next analysed whether anti-64K sera could immunoprecipitate GAD from brain and islets. Membrane and soluble fractions were prepared from brain and islets and immunoprecipitated with serum S3, anti-GAD SMS sera, anti-64K IDDM sera, and control sera. Presence of GAD in the immunoprecipitates was analysed by western blotting. The blots were probed with serum S3 or serum 7673, a rabbit serum raised to a synthetic 17-amino-acid peptide corresponding to the carboxyl terminus of the larger rat brain GAD isoform (ref. 18; and D. Gottlieb, personal communication) (M.S. and A.R., unpublished data). Both sera gave identical results. Figure 2 shows a western blot containing some of such immunoprecipitates probed with serum S3. As expected, an immunoreactive band with the electrophoretic mobility of GAD was detected in immunoprecipitates obtained with anti-GAD sera (Fig. 2, lanes 11, 12, 16). A band of identical mobility was visualized in all immunoprecipitates obtained with

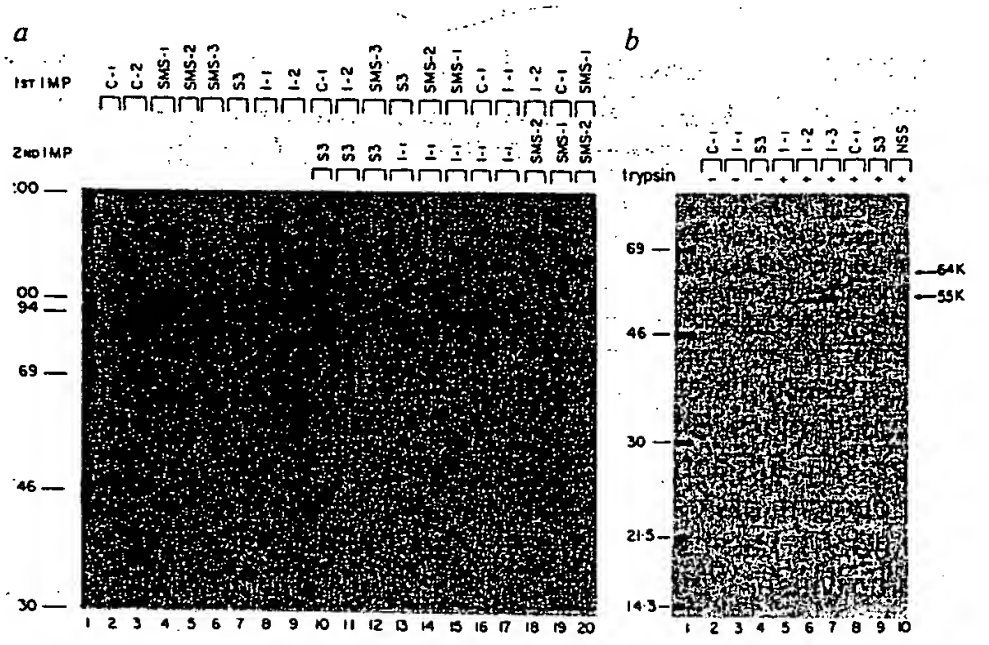


FIG. 1 Anti-GAD sera and anti-64K IDDM sera recognize the same protein in rat islets. *a*, Fluorogram of an SDS-PAGE showing immunoprecipitation of Triton X-114 detergent phase cytosolic fraction from [^{35}S]methionine-labelled rat islets (S-100 DP) with anti-GAD sera, anti-64K IDDM sera and control sera. Lanes 2–9, samples from a single immunoprecipitation with sera indicated at the top of each lane; lanes 10–20, samples from a second immunoprecipitation of supernatants remaining after a first immunoprecipitation. Sera used for the first and second immunoprecipitations are indicated at the top of each lane. Sera used for the immunoprecipitation were: C, sera from healthy individuals; SMS, sera of SMS patients previously shown to be GAD antibody positive[11]; I, sera from newly diagnosed anti-64K-positive IDDM patients[7] (numbers indicate patient code); S3, a sheep antiserum raised to purified rat brain GAD[17]. Relative molecular mass markers are shown in lane 1 ($M_r \times 10^{-3}$). The anti-GAD sera immunoprecipitate GAD from supernatants after immunoprecipitation with control serum (lanes 10 and 19), but not from supernatants after immunoprecipitation with anti-64K sera (lanes 11 and 18). The anti-64K sera immunoprecipitate the 64K protein from supernatants after immunoprecipitation with control serum (lane 16), but not from supernatants after immunoprecipitation with anti-GAD sera (lanes 13–15). The triple band represents the 65K form and 64K α and β forms of the 64K autoantigen (H. Schierbeck, L. Aagaard and S.B., manuscript submitted). *b*, Fluorogram showing immunoprecipitation of the 64K protein and GAD from S-100 DP of [^{35}S]methionine-labelled rat islets before (lanes 2–4) and after (lanes 4–10) trypsin digestion. Serum codes are as in legend for *a*. NSS is a preimmune sheep serum. Trypsin digestion results in a 55K immunoreactive fragment that is recognized by both anti-64K sera and the anti-GAD serum S3.

METHODS. Neonatal rat islets were isolated and labelled with [^{35}S]methionine as described[9]. Islets were swollen on ice for 10 min in 10 mM HEPES, pH 7.4, 1 mM $MgCl_2$ and 1 mM EGTA (HME buffer) and then homogenized by 20 strokes in a glass homogenizer. The homogenate was centrifuged at 2,000*g* to remove cell debris and the postnuclear supernatant centrifuged at 100,000*g* for 1 h to obtain a cytosol (S-100) and a particulate (P-100) fraction. Amphiphilic proteins were purified from the S-100 fraction by a modification of the method described by Bordier[29] for Triton X-114 (TX-114) phase separation. S-100 fraction was made 1% in TX-114, warmed at 37 °C for 2 min to induce TX-114 phase transition, and centrifuged at 15,000*g* for 2 min to separate the aqueous and detergent phases. The detergent phase was diluted in 20 mM Tris buffer, pH 7.4, 150 mM NaCl (TBS) and immunoprecipitated as described[9] using DP from 300 islets for each immunoprecipitate. Immunoprecipitates were analysed by SDS-PAGE (10%) and processed for fluorography[24]. For the trypsin digestion, S-100 DP from 4,000 islets was diluted to 200 µl in TBS. Trypsin (0.74 units) was added and the sample was incubated for 1 h at 25 °C. The reaction was stopped by addition of 10 µl 10 mM HEPES, 5 mM EDTA, 5 mM pyrophosphate, 5 mM benzamidine–HCl, pH 7.5. Digested and undigested material was immunoprecipitated and the immunoprecipitates analysed by SDS-PAGE (15%). Anti-64K sera were from three newly diagnosed IDDM patients[7], control sera were from two healthy individuals. SMS sera were from three anti-GAD-positive individuals[11]. The S3 antiserum was a gift from I. J. Kopin.

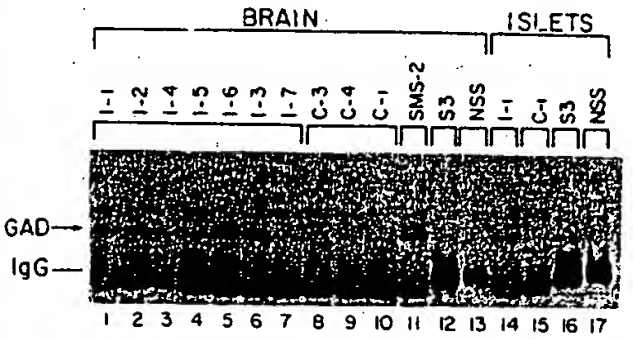


FIG. 2 Anti-64K antibodies immunoprecipitate GAD from brain and islets. Western blot of immunoprecipitates of rat brain (S-100 DP) and islet cell (P-100 DP) fractions obtained with anti-64K sera and anti-GAD sera. The blot was probed with the S3 serum. Sera used for the immunoprecipitation are indicated at the top of each lane by the same codes as in Fig. 1. The 64K protein immunoprecipitated from both brain and islets is immunostained by anti-GAD antibodies. In the gel shown GAD migrated as a single band. METHODS. Neonatal rat brain was homogenized at 4 °C in seven volumes of HME buffer followed by centrifugation at 100,000*g* for 1 h to obtain S-100 and P-100 fractions. S-100 DP was prepared and aliquots (1/13 brain per lane) immunoprecipitated as described in the legend to Fig. 1. P-100 was prepared from neonatal rat islets and extracted in 200 ml TBS with 1% Triton X-114 for 2 h at 4 °C (ref. 9). P-100 DP was prepared as described for S-100 DP and aliquots (1,500 islets per lane) immunoprecipitated. Immunoprecipitates were subjected to SDS-PAGE followed by electroblotting to a PVDF membrane (Immobilon)[30], probing with the S3 serum and visualizing by alkaline phosphatase-conjugated rabbit anti-sheep IgG.

anti-64K sera (7/7) (Fig. 2, lanes 1–7, 14), but not in those obtained with control sera (Fig. 2, lanes 8–10, 13, 15, 17). These results demonstrate that the protein immunoprecipitated from brain and islets by anti-64K sera is indistinguishable from GAD.

64K protein has GAD enzyme activity

If the 64K autoantigen is GAD, then the 64K protein should have the enzymatic properties of GAD. We therefore investigated whether GAD enzyme activity could be removed from islet and brain cell lysates by immunoprecipitation with an anti-64K IDDM serum, and whether GAD activity could then be measured in the immunoprecipitates. Brain and islet cell fractions were immunoprecipitated with increasing amounts of an anti-64K IDDM serum, and the GAD enzyme activity measured after immunoprecipitation in both supernatants and pellets (Fig. 3*a*, *b*). Immunoprecipitation with increasing amounts of anti-64K serum but not with control serum removed the GAD activity from both brain and islet cell lysates in a dose-dependent manner, and, in parallel, increasing amounts of GAD activity appeared in the immunoprecipitates. The GAD activity recovered in the immunoprecipitates did not account for all the activity lost from the supernatants, probably owing to an inhibiting effect of antibodies on enzyme activity. For islet cell extracts from [^{35}S]methionine-labelled islets, SDS-PAGE revealed that the only islet cell protein specifically detected in the immunoprecipitates obtained with the anti-64K IDDM serum was the 64K autoantigen (see Fig. 1). Thus the GAD enzyme activity measured in immunoprecipitates obtained with the anti-64K IDDM serum is a property of the 64K autoantigen.

Brain and β-cell GAD

Analysis of GAD enzyme activity in neonatal and adult rat tissues showed that the expression of GAD is high in brain and islet cells and is either absent or low in a variety of other tissues (results not shown), confirming previous reports[19]. In islets,

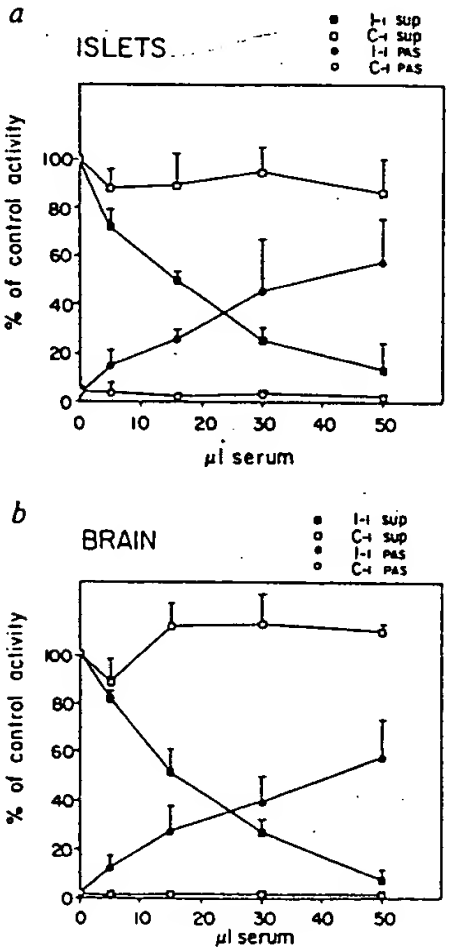


FIG. 3 Precipitation of GAD activity from brain and islets with anti-64K autoantibodies. *a*, Aliquots (700 islets per sample) of S-100 DP from neonatal rat islets were immunoprecipitated with increasing amounts of the anti-64K IDDM serum I-1 (closed symbols) or the control serum C-1 (open symbols). GAD activity was measured in supernatants after immunoprecipitation (squares) and in pellets (circles). The activity in the supernatants was calculated as percentage of the activity in non-immunoprecipitated samples incubated with the same amount of control serum. The activity in the immunoprecipitates was calculated as percentage of the activity in samples incubated without serum. Values are mean of three experiments ± s.d. *b*, As *a*, except that aliquots of S-100 DP from neonatal rat brain were used instead of islet cell material.
METHODS. S-100 DP was prepared from islets and brain as described in the legends to Figs 1 and 2, except that buffers were supplemented with 1 mM aminoethylisothiouronium bromide hydrobromide (AET) and 0.2 mM pyridoxal 5'-phosphate (PLP). S-100 DP was diluted 10 times in 50 mM potassium phosphate pH 6.8, 1 mM AET, 0.2 mM PLP (buffer A) and incubated with the indicated amounts of sera in a total volume of 150 ml for 7 h at 4 °C. Immunocomplexes were absorbed to 150 μl protein A-Sepharose beads (PAS Pharmacia) and isolated by centrifugation. The supernatants were collected and centrifuged three times to remove traces of PAS. PAS pellets were washed five times by centrifugation in buffer A. Enzyme activity in the PAS pellets and the supernatants was measured using a modified version of the assay first described by Albers and O'Brady[31]. Both PAS pellets and supernatants were transferred to 1.5-ml screw cap tubes; 20 μl 5 mM L-glutamate in buffer A and 0.4 μCi [1-^{14}C]L-glutamate (59 mCi mmol^{-1}, Amersham) were added. The tubes were closed immediately with a cap containing Whatman filter paper soaked in 50 μl 1 M hyamine hydroxide in methanol and incubated for 2 h at 37 °C. The filter paper was then removed and the absorbed $^{14}CO_2$ measured in a scintillation counter. The specific activity of GAD in homogenates of neonatal brain and islet cell material was similar (~40–70 mU per g protein).

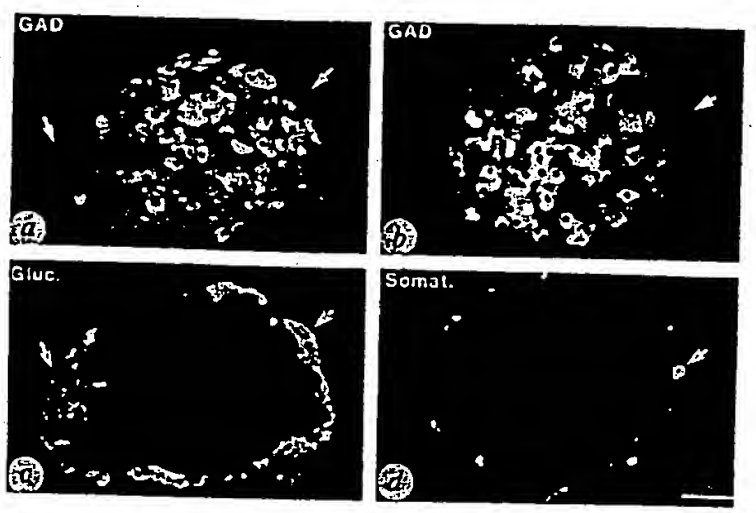


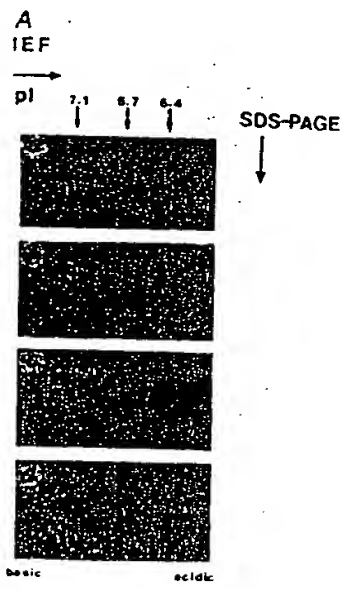
FIG. 4 Immunofluorescence staining of pancreatic islets with GAD, glucagon and somatostatin antibodies. Immunofluorescence micrograph showing pancreatic islets. The islet in *a* and *c* was double-labelled for GAD and glucagon. The islet in *b* and *d* was double-labelled for GAD and somatostatin. Arrows point to corresponding cells in the two pairs of panels. The β cells in the central core of the islet are brightly stained with the GAD antibody, whereas the cells positive for glucagon and somatostatin do not stain with the same antibody.

METHODS. Formaldehyde-fixed frozen sections of rat pancreas were first rhodamine-labelled for GAD using a mouse monoclonal GAD6, raised against purified rat brain GAD[16], and then fluorescein-labelled for glucagon or somatostatin using rabbit polyclonal antibodies to either hormone and using methods described[32]. GAD6 was a gift from D. I. Gottlieb, University of Washington. Scale bar, 25 mm.

double immunostaining with a monoclonal antibody to GAD and either glucagon or somatostatin confirmed the localization of GAD to the β-cell core, and the absence of GAD in the other endocrine cells, which are localized to the islet periphery (Fig. 4). GAD in brain and islets was found to have identical mobility on SDS-PAGE (Fig. 2) and by two-dimensional gel electrophoresis using isoelectric focusing/SDS-PAGE (Fig. 5*a*). We compared the immunoreactive trypsin fragments generated from brain and islet GAD. Trypsin generated a 55K immunoreactive fragment from both islet and brain GAD (Fig. 5*b*; see also Fig. 1). In both tissues GAD was found in a soluble hydrophobic form as well as a membrane-bound hydrophobic form (Fig. 5*b*) as described for the 64K islet cell autoantigen (H. Schierbeck, L. Aagaard and S.B., manuscript submitted). The 65K component of the 64K protein in islets (H. Schierbeck, L. Aagaard and S.B., manuscript submitted) was detected in brain and islet cells in some (Figs 1*a*, 5*A*, *b*, *c*, 5*B* and 6*A*) but not in other analyses (Figs 1*b*, 2). Furthermore, the 64K α/β doublet was detected in some analyses (Figs 1*a*, 6*A*). The immunochemical and biochemical properties of the brain and islet GAD therefore indicate that they are very similar.

Antibodies to GAD in SMS and IDDM sera

The GAD reactivity in SMS sera has been demonstrated by western blotting and by immunocytochemical staining of fixed tissue sections[10,11], that is, assays that involve complete or partial denaturation of the antigen. The standard assay for 64K antibodies in IDDM sera has been immunoprecipitation from islet cell lysates prepared in nondenaturing conditions[4–9]. We selected sera from the individuals who had the highest immunoreactivity to the 64K autoantigen in immunoprecipitation experiments in a survey of 112 IDDM patients and prediabetic individuals without SMS[7] and tested them for immunoreactivity to the brain GAD protein on western blots (five sera) and for immunostaining of GABA-secreting neurons (7 sera). The results were compared with those for SMS sera. In contrast to the SMS sera, none of the IDDM sera detected the denatured GAD protein on western blots (Fig. 6*A*), and only one was able



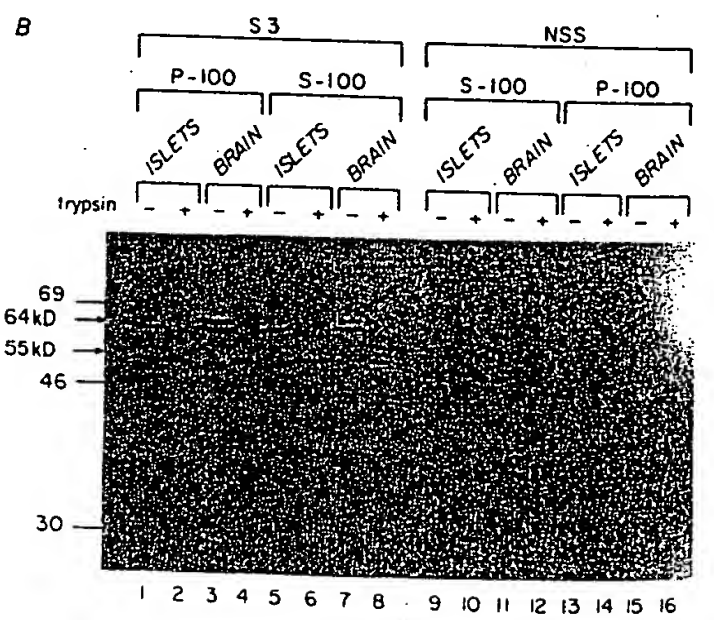


FIG. 5 GAD in brain and islets have similar properties. *A*, Two-dimensional gel electrophoresis of neonatal rat and islet cell GAD/64K antigen. *a*, *b*, Western blots of two-dimensional gels of a neonatal islet particulate fraction (*a*) and a brain fraction (*b*) probed with the GAD antiserum S3. *c*, *d*, Fluorograms of two-dimensional gels of immunoprecipitates of a [^{35}S]methionine-labelled rat islets extract (S-100 DP) with the anti-64K IDDM serum I-1 (*c*) and *a* with a control serum C-1 (*d*). The soluble fractions of brain and islets (*b* and *c*) contain both the 65K pI 7.1 component and the 64K 6.7 α component, whereas the particulate fraction contains only the 64K pI 6.7 α component (H. Schierbeck, L. Aagaard and S.B., manuscript submitted). Both the 65K and the 64K component display charge heterogeneity previously described for the 64K autoantigen in islets[9]. The panels both demonstrate the identical behaviour of the 64K protein (*c*) and GAD (*a* and *b*), further proving they are the same protein, and show the similarities of brain and islet GAD with regard to both charge and size. *B*, Western blot of soluble and particulate GAD from neonatal rat brain and islets before and after trypsin digestion. Lanes 1–8, probing with S3 serum; lanes 9–16, probing with normal (preimmune) sheet serum. Trypsin digestion of both brain and islet GAD results in a 55K immunoreactive GAD fragment.

METHODS. A particulate fraction was prepared from neonatal rat islet homogenates by centrifugation at 36,000*g* (*a*). A low-speed synaptosomal supernatant was prepared from brain as described[33] (*b*). S-100 DP was prepared from neonatal rat islets and immunoprecipitated as described in the legend to Fig. 1 (*c* and *d*). Two-dimensional gel electrophoresis was performed as described by O'Farrell[34] and modified by Ames and Nikaido[35]. Immunoblotting was according to Towbin *et al.*[30]. GAD in *a* and *b* was visualized by probing with the anti-GAD serum S3, followed by rabbit anti-sheep IgG serum and ^{125}I-labelled protein A and autoradiography. For *B*, S-100 DP and P-100 DP from islets and brain were prepared as described in legends to Figs 1 and 2 and digested with trypsin as described in legend to Fig. 1. Brain and islet cell fractions were subjected to SDS-PAGE using 15% polyacrylamide gel. Western blotting and staining procedures were as described in legend to Fig. 2.

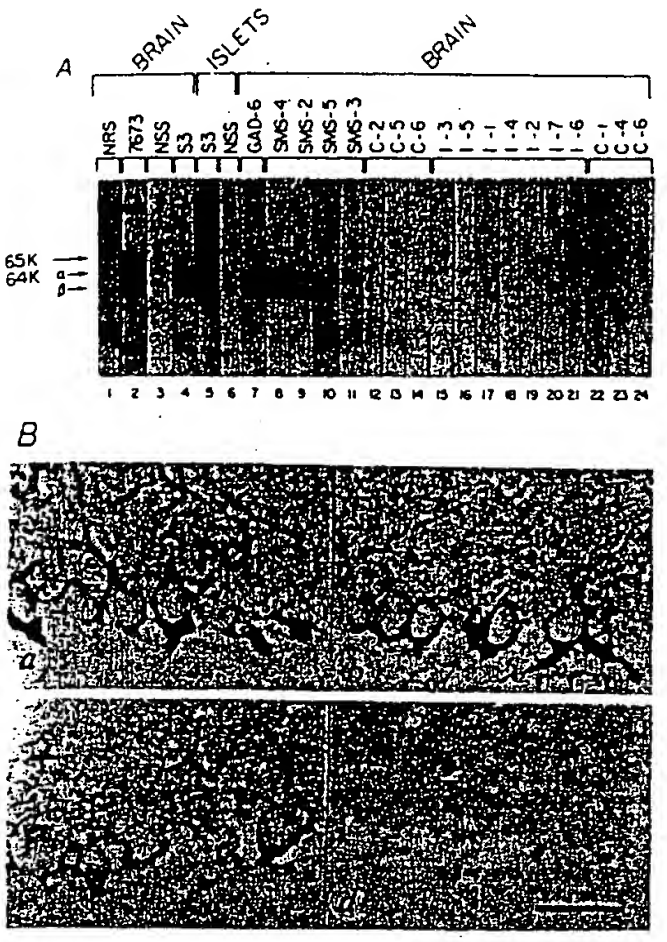


FIG. 6 Comparison of GAD immunoreactivity in SMS and IDDM sera. *A*, Western blots of S-100 DP from neonatal rat brain (lanes 1–4 and 7–24) and islets (lanes 5 and 6) probed with anti-GAD SMS sera and sera from IDDM patients and prediabetic individuals with high immunoreactivity to the 64K/GAD protein in immunoprecipitation experiments. Sera are indicated at the top of each lane by the same codes as in Fig. 1. The S3 serum, 7673 serum, GAD6 serum and anti-GAD SMS sera recognize the denatured form of GAD in brain and islets, whereas the anti-64K IDDM sera do not. The S3 and 7673 sera react with both the 65K form and the 64K α and β forms, whereas GAD6 (previously shown to react selectively with the smaller brain GAD isoform[16]) and the SMS sera only react with the 64K α and β components. *B*, Bright-field light microscopy micrographs showing immunostaining of GABA-secreting nerve terminals in the rat cerebellar cortex with SMS and IDDM sera. *a*, SMS-3 serum; *b*, IDDM serum I-8; *c*, IDDM serum I-2; *d*, IDDM serum I-1. All sera, except I-1, show the typical stain of GABA-secreting nerve terminals. Note the accumulation of immunoreactivity at the base of the Purkinje cells, where the GABA-secreting nerve endings of basket cells terminate.

METHODS. S-100 DP aliquots from rat brain and rat islets were subjected to SDS-PAGE, electroblotted and immunostained as described in the legend to Fig. 2. Dilutions for immunostaining of western blots were 1/200 for GAD6, 1/250 for IDDM sera, control human sera, SMS sera 2 and 5, the 7673 serum and normal rabbit serum, 1/500 for SMS sera 3 and 4 and 1/2,000 for S3 and normal (preimmune) sheep serum. For *B*, methods as described in ref. 11.

to weakly immunostain GABA-secreting neurons (Fig. 6*B*, *c*). Titration of IDDM and SMS sera in immunoprecipitation experiments furthermore showed that the titre of anti-GAD antibodies in SMS sera was 10–200 times that in IDDM sera (results not shown). In another survey of 74 IDDM patients, only three were positive for antibodies to GABA-secreting neurons (Fig. 6*B*, *b*) including the only two which were positive by western blotting (ref. 11, and results not shown). By contrast, all SMS sera that were positive by immunocytochemistry and/or western blotting were also positive by immunoprecipitation (results not shown). So anti-GAD antibodies in SMS patients are generally present at higher titres and recognize more distinct epitopes than anti-GAD antibodies in IDDM patients. However, in rare cases, anti-GAD antibodies in IDDM patients can recognize denatured GAD. Whereas the 7673 and S3 antisera recognize both the 65K form and the 64K α and β isoforms on western blots (Fig. 6*A*, lanes 2, 4, 5), human GAD autoantibodies as well as the monoclonal antibody GAD6 preferentially recognize the smaller 64K α and β isoforms (Fig. 2, lanes 7–11). The GAD6 antibody is specific for the smaller GAD isoform in brain[16].

Discussion

The 64K autoantigen is the enzyme glutamic acid decarboxylase. We have demonstrated that the 64K autoantigen in IDDM is the enzyme GAD in pancreatic β cells. GAD, its product GABA, and the GABA-metabolizing enzyme GABA transaminase are present at high levels in pancreatic β cells as well as in GABA-secreting neurons[19]. GABA may act as an 'endocrine transmitter' in islets[20–23] and this may explain why GAD is expressed in β cells. The β cell-specific expression of GAD in islets is consistent with its being an autoantigen associated with the selective destruction of pancreatic β cells in IDDM. GAD is a major autoantigen in SMS, a disease in which GABA-secreting neurons are thought to be affected[10,11]. Thus pancreatic β cells and GABA-secreting neurons share a protein that is unusually susceptible to becoming an autoantigen, an observation that will surely motivate studies not only into the pathogenesis of IDDM and SMS, but also into the mechanisms of generation of self-tolerance by the immune system, and its failures.

Differences in humoral autoimmunity to GAD in SMS and IDDM. The humoral autoimmunity towards GAD in the two diseases in which GAD-expressing cells are either destroyed (IDDM) or thought to be affected (SMS), suggests a direct relationship between GAD autoantibodies and the diseases. Not all SMS patients with GAD antibodies develop IDDM, and only a small proportion of IDDM patients develop SMS, in spite of their having GAD autoantibodies for several years. This suggests that there are other components to both diseases. The development of SMS seems to involve a stronger antibody response to GAD and includes epitope recognition which is usually absent in IDDM, perhaps as a result of different characteristics of pancreatic β cells and GABA-secreting neurons. For example, the microenvironment of the CNS is protected by the blood–brain barrier and the antigenic requirements to initiate an autoimmune response across this barrier may be distinct. Pancreatic β cells express major histocompatibility complex (MHC) class I molecules[24] which are thought to present self-peptides to the immune system, whereas CNS neurons normally do not[25].

Subcellular localization of GAD in brain and islets and its role as an autoantigen. Synaptic-like vesicles have recently been identified in endocrine cells[26] and may be the storage sites of GABA in β cells, similar to synaptic vesicles in brain. Immunofluorescence studies have shown colocalization of GAD and membrane markers of these synaptic-like microvesicles (A.R., M.S. and P.D.C., unpublished results). We found that GAD in both brain and islets behave similarly with regard to hydrophobicity and compartmentalization, that is, both were detected in a soluble hydrophobic and a membrane-bound hydrophobic form. Although it cannot be excluded that GAD is expressed at the surface of β cells, it is more likely that the protein remains confined to the cytoplasmic space. Thus autoantibodies to GAD are unlikely to see the intact antigen on the surface of normal β cells. But peptides derived from the GAD molecule may be

expressed at the cell surface in the cleft of MHC class I antigens, and so be recognized by pathogenic T cells (ref. 27, and refs therein).

The identification of the 64K antigen as the enzyme GAD is of relevance in elucidating the role of this antigen in the development of IDDM. If the GAD autoantigen is shown to be critical for the initiation of β-cell destruction, then an approach to therapy might be to develop ways of preventing or reversing the autoimmune response towards GAD in β cells of susceptible individuals. This would be similar to the successful prevention of autoimmune disease in experimental allergic encephalomyelitis[28]. □

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